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Clues to the Etiology, Pathology, and Therapy of Cancer Provided by Analogies With Transplantation Disease[1,2,3]

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SUMMARY

The hypothesis is developed that a malignant tumor arises from a single cell that has suffered loss or inactivation of a single gene, if in heterozygous condition, or of both alleles if homozygous. The relevant genes are considered to be those determining the formation of histocompatibility antigens. The precancer cell lacking one (or more in case of multiple gene loss) of these antigens responds to the proliferative stimulus continuously provided by the, now foreign, "host" antigen, in the same manner as do ordinary antibody-forming cells to the administration of a foreign antigen. Production of cell-fixed and, to some extent, of circulating antibodies by the tumor cells is assumed to be primarily responsible for the cancer syndrome, an experimental counterpart of which is represented by the transplantation diseases such as "runt disease." Directions of exploration of therapeutic measures are indicated primarily on the basis of utilization of the principle of allergic damage to cells, and, as more remote prospects, by supplying missing deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), and by autoimmunization procedures.—J. Nat. Cancer Inst. 25: 1197–1229, 1960.

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INTRODUCTION

IT IS the purpose of this article to indicate how a consideration of transplantation disease and other embryological and immunogenetic phenomena may provide a general theory of cancer in terms of loss-mutation and immunological "graft-versus-host" reaction.

Transplantation disease is a generally lethal, wasting condition that arises in certain experimental situations involving the transfer of tissue from one animal to another. One form of this syndrome, known as *"runt disease,"* develops in an animal that has received, in a fetal or neonatal stage, an injection of adult cells from an individual that differs from the host in histocompatibility antigens. A concise account of the phenomenon has been presented recently by Billingham (1959) who, together with Brent (1956, 1957) discovered and named this condition in experiments in which they were attempting to induce immunological tolerance in mice. At about the same time Simonsen (1957) observed this pathological effect of adult homologous cells injected into embryonic or newborn chicks and mice. A similar syndrome, termed *secondary radiation sickness, homologous disease, heterologous disease,* or *wasting disease* develops belatedly in animals that have been protected from acute radiation death by injection of homologous or heterologous hematopoietic cells. It was described by Barnes and Loutit (1954), Cole, Habermeyer, and Bond (1955), Trentin (1956), Makinodan (1956), Congdon and Urso (1957), and van Bekkum and Vos (1957) among others, and is the subject of extensive current investigation. A third situation that can produce a similar ailment consists simply in the injection of *parental* lymphoid cells into a normal young or adult F_1 *hybrid*, as described by Cole and Ellis (1958), Simonsen *et al.* (1958), Trentin (1958, 1959), Boyse (1959), Gorer and Boyse (1959), Nowell and Cole (1959), and Vos *et al.* (1959). Related to this also is the

syndrome termed *parabiosis intoxication* which develops when two animals are joined surgically. When the parabionts consist of an F_1 hybrid and a parental strain animal, it is generally the hybrid that succumbs while its partner remains apparently unaffected, as has been found by van Bekkum *et al.* (1959), Eichwald *et al.* (1959), and Gurney and Taylor (1960). The term *transplantation disease* will be used here to refer to the wasting syndrome arising from any of these experimental situations.

Simonsen (1953) and Dempster (1953) had suggested, from observations of changes in reticuloendothelial cells of transplanted kidneys in dogs, that grafted tissues might react immunologically against their host. This "graft-versus-host" reaction is now the generally accepted interpretation for the pathology of the transplantation diseases. The transplanted homologous or heterologous cells have been shown to persist in irradiated animals (Lindsley *et al.*, 1955; Mitchison, 1956; Ford *et al.*, 1956; Nowell *et al.*, 1956; Makinodan, 1956, Smith *et al.*, 1957; Gorer and Boyse, 1959), and the delayed death has been attributed (Trentin, 1957, Cole and Ellis, 1958, Uphoff, 1958) to the immunological response of these cells to host antigens. Failure of the host to destroy the foreign cells is attributable to immunological immaturity with development of "tolerance" (Billingham, Brent, and Medawar, 1956) in "runt disease," to impairment or destruction of host immunological reactivity in secondary radiation sickness, to lack of foreign antigens on the part of parental cells injected into an F_1 hybrid host and in parent-hybrid parabiosis, and to immunological paralysis in some homologous or heterologous combinations. A single gene difference at the *H-2* locus (which is apparently the most important one involved) is sufficient for secondary disease to develop in irradiated mice "protected" with homologous bone marrow (Uphoff and Law, 1959).

Apart from the impairment of growth in "runt disease," these various transplantation diseases all exhibit a similar general pathology that involves delayed onset, emaciation, skin lesions, poor condition of the fur, diarrhea, anemia, and initial enlargement followed by involution of the lymph nodes and other lymphoid tissue. The runting effect can, of course, only be exhibited when fetal or not fully grown animals are the recipients. The syndrome of the transplantation diseases exhibits features commonly noted in various neoplastic diseases. Even frank tumors may develop, as in experiments by Uphoff and Law (1958) who found a high incidence of malignant lymphomas of *donor-cell* origin in lethally irradiated hybrid mice that were protected from acute radiation death by parental bone marrow, and in the experiments of Vos *et al.* (1959) who obtained lymphomas in nonirradiated F_1 mice that had received parental cells.

The present thesis is that, as in the transplantation diseases, the malignant nature of the neoplastic diseases is due primarily to an immunological response of some, or all, of the cancer cells against "host" tissue, and that this response occurs because the cancer cells lack one or more "host" transplantation antigens, this lack being occasioned by inactivation or loss of the corresponding gene(s).

An immunological theory of carcinogenesis has been developed by H. N.

Green (1958, 1959), who proposes that carcinogenic chemicals, viruses, or radiation induce a change of cytoplasmic tissue-specific antigen to autoantigen with a resulting local immune reaction. Persistence of the reaction in repeated exposure to carcinogen, or promoting agent, is assumed to result in a strain of cells lacking the specific antigen as an adaptation to continuous attack by a nonlethal antibody. He interprets the aggressive properties of the cancer cell as an immunological attack of the tumor on the host. The role of immunological processes in cancer has been emphasized also by Cajano (1959, 1960). Kaplan and Smithers (1959) have recently proposed that homologous disease is the experimental counterpart of Hodgkin's disease in man. They suggest, too, that the loss of *tissue-specific* antigens, as indicated in the experiments of Weiler (1959) on hepatomas and kidney carcinomas, would enable the lymphoid tumor cells to react against and destroy the patient's normal lymphoid and other hematopoietic cells. Ira Green *et al.* (1960) have further suggested that patients with Hodgkin's disease may be chimeras resulting from maternal lymphocytes traversing the placenta and remaining temporarily dormant in the offspring. The present account represents further exploration, along somewhat different lines, of the possibility of coordinating some of the current knowledge into a general theory of cancer. The principal parts of such a general theory are: 1) inactivation or loss, in the precancer cell, of gene(s) determining the presence of histocompatibility antigen(s), 2) stimulus to proliferation due to exposure to lost antigen(s) present in the "host," 3) antibody formation leading to physiological impairment and destruction of "host" tissue.

Gene loss or inactivation of a different sort has been assumed in other theories of cancer, particularly by Potter (1958) and Osgood (1959). Haddow (1958) has emphasized that gene-loss is the most likely effect of chemical carcinogens and has related this to immunological and other theories. The present theory does not require that the metabolism, proliferative capacity, or other properties, be modified intrinsically by gene-loss, by mutation, or by carcinogenic virus. It accords, then, with evidence (*cf.* Weinhouse, 1955; Lajtha, 1957; Paul, 1960) indicating that the neoplastic *cell* may not be essentially different in such properties, and structure, from the corresponding normal cell.

GENE LOSS OR INACTIVATION AND CHROMOSOMAL ABERRATIONS

General Statement

Several early investigators, notably Boveri (1929) and Winge (1930), have proposed that chromosomal changes commonly seen in tumors are causally related to the neoplastic properties of the cell. According to the present thesis the neoplastic state is initiated by gene loss or inactivation, but not by mutation to an allele that determines the formation of a new

histocompatibility antigen. The latter process, even if occurring in immunologically competent cells, would result in both the "host" cells and the altered cells reacting immunologically against each other, with the "host" likely to win out. Possibly benign tumors and tumor regression may result from such a process, but the malignant state is considered to be the result of loss or inactivation.

Inactivation or loss of a gene, or group of genes, may well occur without any visible chromosomal change. One would not, then, anticipate that all neoplastic cells should exhibit chromosomal aberrations. However, the occurrence of chromosomal aberrations, involving deficiencies in particular, would be expected to be associated with the assumption of malignant properties. In inbred animals the deficiency, or inactivation, would have to be homozygous. In animals heterozygous for the presumed histocompatibility genes, a single loss or inactivation should suffice. The altered cell or cells are tolerated by the "host" since they possess no foreign antigens, but they in turn recognize the "host" as foreign and, if immunologically competent, they respond to "host" antigens. The question of the kinds of cells that are immunologically competent, or that can become so, is discussed further in the section "Reversibility of Differentiation."

Chromosomes of Malignant Cells

From the extensive literature on the chromosomes of malignant cells, one can find many examples of no visible alterations, but the most common situation is one of considerable change. The subject has been frequently reviewed (*e.g.*, Koller, 1956; Hauschka, 1957, 1958; Levan, 1956; Makino, 1957; Hsu, 1959). A few recent studies may be cited here.

Baikie *et al.* (1959) found abnormalities in number and/or in form of chromosomes in 4 out of 5 patients with acute leukemia, but none in 6 with chronic leukemia and 2 with myelomatosis. Makino *et al.* (1959) found chromosome numbers ranging from hypodiploid to hypertetraploid in 30 human tumors. Ford *et al.* (1958) examined cells from the primary growths or first transplants of more than 60 mouse leukemias and found chromosomal aberrations in about 90 percent. However Ohno, Kovacs, and Kinosita (1959), Nowell *et al.* (1958), and Kaplan (1959) reported predominantly normal numbers in spontaneous and induced mouse and human neoplasms, as did Bayreuther (1960) in an extensive study of spontaneous and induced (by viruses or chemicals) primary tumors of the domestic fowl, mouse, rat, rabbit, Chinese hamster, cattle, and man. In primary, methylcholanthrene-induced fibrosarcomas of the mouse, Hellström (1959) found the diploid chromosome number in the stem cells of 4 mice and aneuploids in the hyperdiploid, triploid, or hypertriploid region in 12 others. In milk-agent-induced primary mammary carcinomas, Tjio and Östergren (1958) found the majority had a modal number of 40 (the diploid number in the mouse), but one of the chromosomes was heteropyknotic in all tumors except one that had 39 chromo-

somes. The heteropyknosis has more recently been reinterpreted by Ohno and Hauschka (1960) as due to allocycly of one of the X chromosomes occurring in normal, as well as neoplastic, diploid female cells.

Gläss (1960) found about 60 percent of aneuploids in butter yellow-induced hepatomas of the rat. The chromosome numbers ranged from 6 to 192 with no indications of grouping about multiples of 21, the haploid number. Hsu and Klatt (1959) observed in initial cultures of rat hepatomas about 60 percent of $2n$-3 chromosome numbers and 40 percent of $4n$-6. Yosida (1959) developed a subline of Yoshida sarcoma of increased transplantability that still had 40 chromosomes, but was grossly distinguishable from the original line by the presence of two unusually large V-shaped and one J-shaped elements.

In the rabbit papilloma, Palmer (1959) found mainly the diploid number, but increasing hyperploidy accompanied transformation to malignancy.

Interpretation of Aberrations

The more recent work, with improved techniques, confirms most earlier accounts of the general occurrence of chromosomal aberrations of aneuploid nature in tumors. While it has also provided additional examples of the normal diploid number of chromosomes occurring in some tumors, the reservation must be made that some or all of these may be aneuploids. For example, in the Chinese hamster, in which the chromosomes are more readily identifiable than in other mammals, Tonomura and Yerganian (1956) have reported finding such pseudodiploids that were actually trisomic-monosomics. One interpretation of the chromosomal aberrations, according to the present thesis of gene loss or inactivation, would assume that, even in tumors with hyperploid stem cells, there are deficiencies. Their aneuploid nature could well be an indication of this. Another interpretation would assume that gene inactivation occurs in the hyperploids by virtue of imbalance. There are many examples, which might be cited from the genetic literature, of phenotypic effects caused by aneuploid addition of chromosomes or fragments thereof. Possibly certain combinations of additional chromosomal material may result in inactivation of the histocompatibility genes in a manner analogous, for example, to the change in phenotypic expression of sex with changes in ratio of number of X chromosomes to number of certain autosomes and the Y chromosome (in mammals). Essentially this implies the existence of modifying genes for the histocompatibility genes. Clear evidence for this does not seem to be available. Since the genes for cellular antigens express themselves co-dominantly with only occasional and perhaps otherwise interpretable evidence of interaction even between the alleles (Irwin, 1949; Cohen, 1960), it does not seem likely that they would be affected by this kind of imbalance, but the possibility cannot be ruled out entirely. Another way of obtaining gene inactivation without loss of chromosomal material would be by the so-called position-effect, discovered originally by Sturtevant (1925), whereby inversion of parts of chromosomes or trans-

locations can result in alteration or lack of the phenotypic expression of the transposed genes. One interesting category occurs generally when genes normally located in euchromatin are transposed close to heterochromatic regions of the chromosomes (Schultz, 1956, 1958). The inactivation is dependent upon many internal and external factors so that the somatic variegation, whereby it expresses itself, may vary. Such position effects are paralleled by the surprising consequences of Y-chromosome hyperploidy, and Cooper (1956, 1959) has presented an explanation of both position and hyperploid effects, including a view of heterochromatin, which accounts for effective inactivation of genes without either genic loss or change.

From the evidence of the occurrence of normal as well as abnormal chromosomal constitution in tumor cells, it would appear that the postulated gene inactivation or loss may occur by any one of these methods: deletion, imbalance, position-effect, or "point" mutation. However, the predominance of chromosomal aberrations in tumor cells, the indications that imbalance is unlikely to affect histocompatibility genes, and the occurrence of hypodiploids, along with indications of deficiencies in the hyperploids, would tend to favor gene loss by deletion, and possibly inactivation by position-effect, as the main processes responsible for the loss of antigens on the part of the tumor cell. The various carcinogenic agents, including viruses, are assumed, then, to act by one or another of these methods of producing gene loss or inactivation. For viruses, the most likely method would seem at present to involve interference with the activity of specific genes by the viral nucleic acid. However, it is possible (*cf.* Roizman and Roane, 1960) that some do not actually infect the tumor cell itself, but by particular destructive action on other tissue cells may set up a carcinogenic condition.

Hsu (1959) has noted that, perhaps coincidentally, rat tumors commonly show losses of chromosomes along with numerous structural changes, whereas most mouse tumors show an increase. Possibly this relates to the inbred nature of the mouse strains studied and consequent need for a homozygous deficiency, or inactivation, to initiate the neoplastic state. Conditions that would affect the two homologous chromosomes would be expected to affect many others and produce hyperdiploid along with hypodiploid cells. The latter, with homozygous chromosomal deficiencies, would be expected to be mostly inviable or of low reproductive capacity, while the hyperploids could contain a sufficient assortment of genes derived from fragments of the deficient chromosomes, as well as possible equivalents in other chromosomes, to permit adequate functional activity. In tissue culture, Tjio *et al.* (1959) found that human cells deficient in one chromosome (one of the X's) give the same plating efficiencies as do normal cells with a full chromosomal complement. Of course, in the whole organism, symbiotic relationships between tissues may permit grossly deficient cells to survive.

The great diversity of chromosome numbers and patterns even among histologically similar tumors is often proposed as an argument against the

view that the aberrations constitute a cause, rather than simply a consequence, of adoption of malignant properties by the cell. Since it is gene loss or inactivation that is considered to be the essential feature, the chromosomal variation seems more readily understandable. Thus inactivation due to genic imbalance could occur with a great variety of chromosomal patterns exceeding a certain minimum of imbalance. Simple gene loss by deletion should presumably permit even greater chromosomal variations without restoration of the postulated histocompatibility antigens. Also on the assumption of inactivation attributable to "position-effect" there could be considerable variation in number of chromosomes without restoration of the ability of the cell to produce the missing antigen. Thus, the objections raised by the occurrence both of normal and abnormal chromosomal constitution are resolved on the basis that the requisite gene loss, or inactivation, may occur with or without cytologically visible chromosomal change. At the same time the occurrence of chromosomal changes involving deficiencies in particular would be likely to result in the development of a neoplasm.

Chromosomes of "Normal" Cells

While cells taken directly from the organism generally show relatively little variation in chromosome numbers, considerable change has often been noted in tissue culture (*cf.* Levan and Biesele, 1958; Ford, 1959). Some of the reported cases of abrupt changes are evidently now attributable to contamination by other cell strains of the laboratory (Rothfels *et al.*, 1959). Also, it is apparently possible to maintain cell strains in culture without change in the normal diploid chromosomal constitution (Tjio and Puck, 1958). Of special interest is the finding that certain originally normal cells, showing aberrant chromosome constitution in tissue culture, have the capacity to initiate tumors (*cf.* Sanford *et al.*, 1956; Levan and Biesele, 1958; Rothfels *et al.*, 1959). This has provided further support for chromosomal theories of carcinogenesis.

FREQUENCY OF SPONTANEOUS AND INDUCED NEOPLASMS IN INBRED STRAINS AND HYBRIDS

Theoretical Expectations

Since the postulated antigenic loss would require double gene loss, or inactivation, in homozygotes, it may be expected that the incidence of spontaneous or induced neoplasms would be much less in them than in heterozygotes. Also assuming that each gene loss, or inactivation, occurs generally by a one-hit mechanism, one would expect (apart from divergence at high dose) the frequency of induced cancer to be a linear function of dose of carcinogen in the heterozygotes and to be proportional to the square of the dose in the homozygotes. Complete homozygosity is, of course, difficult to attain even after extensive inbreeding. How-

ever, for the present purpose, strains within which reciprocal skin transplantation can be made will be termed homozygous. Animals that are heterozygous at one or more histocompatibility loci may also be homozygous at other loci. This introduces a two-hit component into the dose-incidence relation of the heterozygotes, the effect being manifest at increasingly higher doses, the fewer the relative number of homozygous histocompatibility loci.

Because of X- and Y-chromosome differences, males of an inbred strain may well be heterozygous for the relevant genes, and they are haploid for genes in the X chromosome that are not represented in the Y, and vice versa. Several recent experiments (*cf.* Eichwald and Silmser, 1955, 1956; Billingham and Silvers, 1959; Zaalberg, 1959) on skin transplantation have demonstrated in mice the occurrence of histocompatibility due to an antigen determined by the Y chromosome. According to Billingham and Silvers (1959), different females may or may not react to this histocompatibility antigen which is much weaker than that determined by genes at the *H-2* locus.

Cancer Incidence in Males and Females

Extensive data are not available on the tumor incidence in the 2 sexes of inbred lines except where endocrinological or milk-agent factors are likely to be involved. A few examples from the literature may serve to illustrate the diversity of results that have been obtained and the corresponding difficulty in reaching a definitive conclusion concerning the possible carcinogenic role of gene loss or inactivation in the X or Y chromosome.

In inbred mice Andervont and Edgcomb (1956) found a higher incidence of methylcholanthrene-induced papillomas in the males than in the females in strains BALB/c, C3H, I, and RIII, and the reverse in strain C57BL, the latter involving the fewest mice. Upton *et al.* (1958) observed in inbred (RF) mice a higher susceptibility of males than of females to radiation-induced myeloid tumors, but the reverse for the thymic lymphomas. Earlier Kaplan and Brown (1952) found the incidence of radiation-induced lymphomas to be higher in female (58%) than male (47%) inbred mice (C57BL). Little (1939) noted in 2 inbred strains of mice that spontaneous tumors were more frequent in the males than in the females, while in the hybrid the situation was the reverse. Heston and Dunn (1951) found no significant sex difference in the induction of tumors in transplants of lung tissue from 2 inbred strains to the F_1 hybrid. Among noninbred animals, the data for humans show, according to Shimkin (1959), no significant sex difference in the incidence of neoplasms common to both sexes (*cf.* Boyland, 1958; Lombard, 1959).

Cancer Incidence in Hybrids

Reviewers (*cf.* Heston, 1951; Burdette, 1955; Law, 1954, 1959; Little, 1959) of the subject of genetic susceptibility to spontaneous and induced

tumors note that the incidence in the hybrid is usually intermediate between the parental strains, when allowance is made for such factors as milk-agent effect, maternal resistance factor, etc. However, there have been many reports of increased incidence. In one of the earlier examples of this, Little (1939) found a tumor incidence of 60 percent in the F_1 hybrids of a cross between an inbred strain of the wild mouse *Mus bactrianus* and an inbred strain of the domesticated *M. musculus*, whereas the incidence in the parents was only 4 and 16 percent, respectively. Other examples of increased incidence of spontaneous and also induced tumors in hybrids of inbred strains of mice have been reported by Andervont (1940, 1943), Warner *et al.* (1945), Woolley *et al.* (1952), Andervont and Dunn (1953), Kaplan *et al.* (1956, 1958), Marchant (1957, 1959), and Richardson and Hummel (1959).

There are also many experiments (*e.g.*, Heston and Dunn, 1951; Shapiro and Kirschbaum, 1951; Trentin and Gardner, 1958; Gilman, 1959) in which the F_1 is approximately as susceptible as the more susceptible parent to spontaneous or induced tumors. While these may not outweigh the many examples that may be cited of intermediate tumor incidence in hybrids, the occurrence, particularly of the higher frequencies, is not readily interpretable on the basis of the usually assumed multiple genes for susceptibility or resistance. If the higher frequencies in hybrids are interpreted in accordance with the postulate of gene loss or inactivation, then the incidences intermediate between the 2 parents, or equal to the high parental strain, would seem to require special explanation. These occur particularly in matings of high by low incidence strains. One may then assume that the selective inbreeding for high cancer incidence entails the accumulation of (*a*) genes that directly or indirectly affect the activity of the particular tissues that give rise to the characteristic tumors of the strain, and (*b*) certain "mutator" genes that affect the expression of other genes, or induce them to mutate (*cf.* Demerec, 1937; Neel, 1942; Rhoades, 1943; Mampell, 1943; Ives, 1950).

The best-known examples of such "mutator" genes are ones inducing chromosomal aberrations. In *Drosophila*, Sturtevant (1929) discovered genetically determined elimination of chromosomes in the somatic cells, and Stern (1936) found genetically determined somatic crossing over. Beadle (1932) described a simple recessive gene in maize that induced "stickiness" of the chromosomes in the somatic, as well as the meiotic, divisions, resulting in nondisjunction and translocation along with apparent gene mutation. He suggested that many deficiencies would be produced. Probably the most extensive examples of genetically controlled production of aberrations have been described by McClintock (1956) in maize. These include deficiencies, duplications, inversions, translocations, and ring chromosomes. Some of the accompanying somatic mutational effects are attributable to homozygous deficiencies. These examples, along with the common occurrence of chromosomal aberrations, including "stickiness," in tumors (Koller, 1954) provide one kind of chromosomal basis for the properties of the high tumor strains.

The expected higher cancer susceptibility of heterozygotes, because of the single-hit requirement, may well be obscured in those cases in which such genetically determined chromosomal effects occur in one or both of the parents. This analysis implies, then, that more weight should be given to the results obtained with hybrids derived from strains of low cancer incidence. Since there are a number of different histocompatibility loci, at least 15 in the mouse, there should be correspondingly greater incidence of spontaneous and induced cancer in the hybrids, the greater the genetic differences between the 2 parental strains. The large effect obtained by Little (1939), crossing 2 species of mice, may be an expression of this rather than of a disharmony of the 2 sets of genes assumed in the usual interpretations. Possibly, too, the high incidence of melanomas obtained (Gordon, 1959), by crossing certain species or strains of tropical fish, may be a further illustration of this effect.

Dose-Incidence Relation

A linear relationship between dose of radiation and incidence of leukemia in human beings appears in the data from the Hiroshima-Nagasaki atomic-bomb survivors and from individuals treated for ankylosing spondylitis, as analyzed by Court-Brown and Doll (1956, 1957) and by Lewis (1957). Experimentally, a linear relationship has also been obtained recently by Bond *et al.* (1960) for radiation-induced mammary-gland tumors in Sprague-Dawley rats over the range from 25 r to 400 r. The line passes through zero incidence at zero dose, but above 400 r the points deviate. The latter is interpreted on the basis of such factors as cell lethality and interference with ovarian function at the higher doses. Objections that have been raised (*cf.* Mole, 1958; Wald, 1958; Brues, 1958) to the human data would not seem to apply to the experiments on the rat. Since both humans and Sprague-Dawley rats are noninbred, the linear relation accords with the expectation based on a single-gene loss or inactivation requirement for heterozygotes.

Experimental dose-incidence data are also available for radiation-induced leukemia in hybrid (LAF_1) mice (Furth *et al.*, 1954), but these do not fit a linear relation. In inbred strains of mice, the relation is (Upton *et al.* 1954, 1958; Kaplan and Brown, 1952) nonlinear. Kaplan and Brown's (1952) data show that the incidence of thymomas in C57BL mice is approximately proportional to the square of the dose.

Hybrid and noninbred mice have been used in experiments with bone-seeking radioisotopes (Sr^{89}, Sr^{90}, Ca^{45}, Ra^{226}) singly or repeatedly injected (Brues, 1949; Finkel, 1958; Finkel and Biskis, 1959). These have been interpreted as showing a nonlinear relation (a quadratic relation is suggested by Burch, 1960) between the incidence of bone tumors and total-radiation dose, expressed usually in terms of the total amount administered. The statistical treatment of some of the data (Finkel, 1958) has been criticized by Kamb and Pauling (1959), and a recent reanalysis (Archer and Carroll, 1960) indicates a linear relation.

Reservations (*cf.* Wollman, 1955; Mole, 1958) apply also to the interpretation of the dose-incidence relation in many experiments with chemical carcinogens in which the procedure is to administer the agent over a prolonged period, for example (Druckrey, 1959), until the first tumor appears. Thus, the finding of Charles and Luce-Clausen (1942), that in inbred C57BL mice the number of papillomas induced by benzopyrene is proportional to the square of the time (as a measure of dose), cannot be taken as strong support for a two-hit mechanism. An analysis, by Heston and Schneiderman (1953), of pulmonary-tumor induction by single injections of dibenz[*a*,*h*]anthracene in inbred strain A mice shows a linear relation, indicative of a single-hit phenomenon, when allowance is made for the spontaneous incidence and for a factor representing reduction in effective concentration, due perhaps to loss in the circulation. It has been suggested (Wollman, 1955) that because of the low solubility of this agent and the tendency of the larger granules to become localized in the lung, the relation to dose may reflect primarily differences in number of large granules in the injected suspensions.

In summary, then, there is some support, derived especially from the experiments of Bond *et al.* (1960) and of Kaplan and Brown (1952), for the thesis of single action for heterozygotes and double action for homozygotes in tumor induction. At the same time there does not seem to be clear-cut evidence to refute this idea. Brues (1949, 1958) has considered the existence of a latent period that varies inversely with dose to be an argument against a one-hit mechanism. Where the latent period refers to the first appearance of a tumor, this can have a statistical explanation based on the decrease in number of animals that develop tumors as dose is decreased. The increase in minimum latent period of this type, as the dose is decreased, would be an expression of the smaller probability of finding the extreme values in the smaller statistical assemblages than in the larger ones.[4] Where the latent period refers to the time at which a large fraction (the mean or 20%) of the animals develop tumors, a decrease with increasing dose may be interpretable on the basis of increasing occurrence of multiple tumor initiation. Unfortunately, this is not readily detectable in the leukemias and is often obscured by metastases in other neoplasms.

With regard to the occurrence of a latent period in general, many factors may contribute to variation in the time required for a tumor to become visible. Possibly some of these may be dose-dependent and thus obscure the primary dose-tumor-initiation relation; for example, effects of a particular carcinogen on the activity of an endocrine organ whose secretions affect the activity of some other tissue in which carcinogenesis is being studied. Allowance must then be made for such effects when they occur, or their absence must be demonstrated. In other situations it may be assumed that there will be variations in the time at which conditions

[4] Professor Robert P. Dilworth, of the Department of Mathematics, California Institute of Technology, confirmed this interpretation of the reported increase in minimum latent period with decrease in dose of carcinogen, in terms of the decrease in number of animals that develop tumors. He derived the expression $\mu - \sigma\sqrt{2 \log n}$ for the mean of the minimum of a sample of size n from a normal population with mean μ and standard deviation σ.

(*e.g.*, attainment of stimulating concentration of host antigens, proper concentration of particular hormones, changes in local environment) become suitable for the multiplication of the cells, but that the proper conditions will occur sooner or later within the organism's lifespan so that affected cells, or a constant fraction thereof, will be able to express themselves as tumors.

LOSS OF ANTIGENS BY TUMOR CELLS

Histocompatibility Antigens

Recent experiments have provided a number of examples of the absence of one or more "host" antigens in tumors, or at least in some of the cells thereof (*cf.* Klein and Klein, 1959; Hauschka, 1957; Gorer, 1956; Sachs and Feldman, 1958). Some experiments by Klein and Klein (1958, 1959) demonstrate this clearly with regard to the histocompatibility antigens of tumors that were induced chemically in F_1 hybrids of certain inbred lines of mice that differed primarily at the *H-2* locus. Generally such tumors, like normal tissues, can be successfully transplanted to genetically identical F_1 hybrids, but not to the parental strain mice. However the Kleins found that 8 out of 23 of these tumors could also grow in one of the parents, but not in the other parent. This means the *H-2* histocompatibility antigen of the latter parent was absent from the tumor. The Kleins favor a mutational change, occurring in some cells of the tumor, as responsible for the antigenic loss. According to the present thesis, loss or inactivation of one of the parental histocompatibility genes occurring in the precancer cell would account for its neoplastic behavior in the *hybrid*, but growth as a tumor in one of the *parents* would require additional gene loss or inactivation.

The Kleins also found 2 hybrid tumors that could grow in both parental strains and in other isogenic strains possessing other alleles of this histocompatibility (*H-2*) locus. These would represent loss or inactivation in the hybrid of both of the *H-2* genes at this locus. According to the present thesis, those hybrid tumors that remain specific for the hybrid would also have suffered gene loss or inactivation but at some other locus. Since there are at least 15 histocompatibility loci in the mouse, extensive testing might well be required before a strain would be found in which the parental-strain-incompatible hybrid tumors would be accepted. According to the present thesis the tumor tissue should behave like normal, nonmalignant, isologous tissue when transplanted into a strain whose genotype is the same as that of the tumor.

Human Blood Group Antigens

In humans the loss of group A and B antigens in tumors of the urinary tract has been reported by Kay (1957). The antigens may not be completely lost, since they are demonstrable in some cells to some degree.

In human leukemic leukocytes Seligmann *et al.* (1955) reported that only 2 antigens could be detected in gel-diffusion tests with rabbit antiserums instead of the 3 found in the normal cells. Additional evidence of antigenic loss has been obtained by Milgrom *et al.* (1959) by complement-fixation tests. Salmon *et al.* (1958) found red blood cell mosaicism with a ratio of 75 percent A_1 cells and 25 percent O cells in a leukemia patient. Other blood factors (M, N, P, Rh, Duffy) are the same for the 2 kinds of cells and, judging from the parental blood types, there is little likelihood that there may have been an unobserved twin, *in utero*, from which blood-forming cells might have been transferred to give rise to the O cells, as originally described by Owen (1945) for cattle. Since the saliva is group A and about as strong as is usually found, and since this individual is heterozygous AO, it appears that a loss of the A gene occurred in the stem cell from which about 25 percent of the circulating erythrocytes was derived. Presumably, according to the present thesis, the leukemia was due to the activity of the leukocytes that were derived from the same stem cell and possessed the same antigenic loss.

Other cases of leukemia in which mutation has been suggested (van Loghem *et al.*, 1957; Stratton *et al.*, 1958; Gold *et al.*, 1959; and Salmon, 1959) involve either (*a*) a change, along with occurrence of the disease, from A antigen to "weak" A, which does not agglutinate in typing serum but still absorbs and elutes antibody, or (*b*) the presence of a mixture of A and "weak" A red blood cells. An alternative interpretation would be that mutation had not occurred in the precursors of the red cells, but rather that gene loss or inactivation had occurred in a lymphoid stem cell and that the antibodies subsequently produced, in response to the "host" antigens, coated all or most of the red cells. In this connection it was observed that the red cells gave a positive Coombs' test for antibody globulin on their surface (van Loghem *et al.*, 1957) and that on injection of tagged A and O red blood cells, the former showed greatly shortened survival (Gold *et al.*, 1959).

Atwood (1958) demonstrated the occurrence of small proportions (around one per thousand) of O or B erythrocytes in the blood of normal A or AB individuals. He also found that A_1 and A_1B individuals possess exceptional cells having the phenotype of subgroup A_2 or A_2B. He suggested that they may be phenocopies or may result from somatic mutation. Where they represent a loss of, rather than a change in, antigens, the stem cell would be potentially neoplastic according to the present hypothesis. However, there are various factors, such as the occurrence of the change in a terminal type of cell, that may be responsible for the failure of these cells to become malignant. More information will be needed before it can be stated whether this situation provides evidence for or against the present views.

Tissue-Specific Antigens

Loss of antigens of a different type, the tissue-specific antigens of liver and of kidney, has been reported by Weiler (1959) in tumors arising in

these organs, and studied primarily by the fluorescent antibody technique. This conclusion, however, is disputed by King *et al.* (1958, 1959) who found that normal rabbit globulin conjugated with fluorescein gave the same differential staining as did the conjugate with globulin from an immunized rabbit. They considered the difference to be due primarily to a greater affinity of the proteins of normal tissues for the acidic fluorescein-globulin conjugate.

Resolution of this difference of opinion concerning whether or not loss of a specific tissue antigen occurs in tumors does not at the moment seem essential to the development of the present thesis. This type of antigen may not be causally related to carcinogenesis in the way that is assumed for the histocompatibility antigens. The latter are common to most, if not all, tissues of the organism, and cells that have lost such an antigen find themselves in the presence of now foreign cells of the "host"; they are in the same situation as are cells of an inbred parent transplanted to the F_1 hybrid. Loss of a tissue-specific antigen would not *per se* be expected to cause the cells to respond immunologically against "host" tissue, since, by definition, the various tissues of the organism normally differ in these antigens.

Present evidence (Medawar, 1959) indicates that histocompatibility antigens are of mucopolysaccharidic nature. However, it is not known whether or not they are represented by the same chemical substance in all tissues in which they occur. It is also possible for one and the same molecule to possess two or more kinds of antigenic determinants. As an analogous illustration, a molecule of antibody globulin may be characterized by antigenic determinants characteristic of the species of animal and by other chemical structures characterizing it as a specific antibody. There may, then, be instances in which loss of some tissue-specific antigen may accompany loss of histocompatibility antigens.

EVIDENCE OF ANTIBODY FORMATION BY NEOPLASTIC CELLS

Antibodies and Cancer Pathology

In addition to the examples noted above of the appearance of "weak" A antigen in patients with leukemia, there are some other indications of the production of antibodies by tumor tissue against "host" antigens.

One of the most common features of the pathology of cancer is the occurrence of anemia. Evidence has accumulated (*see* review by Price and Greenfield, 1958) that the anemia is generally of the hemolytic type and that red cell antibodies can be detected by hemagglutinative, hemolytic, and direct Coombs' tests (Hyman and Harvey, 1955; Dacie, 1955; Frumin and Kohn, 1955; Rosenthal *et al.*, 1955; Chodos *et al.*, 1956; Price *et al.*, 1959). Green *et al.* (1957) reported that 43 percent of 451 cancer patients were positive in the direct Coombs' tests as contrasted with 15 percent of 241 noncancerous patients. Adelsberger and

Zimmerman (1959) found hemolysins in extracts of 5 out of 25 human lung tumors tested.

Besides the indications of red blood cell antibodies, there is also evidence of platelet agglutinins in thrombocytopenic leukemia, as described by Dausset (1959). It seems likely that these antibodies would be involved in the hemorrhagic conditions that characterize the pathology of many forms of cancer.

It is, of course, not a necessary consequence of the thesis of antihost-cell immunological activity on the part of tumor cells that the antibodies be detectable at all times. Also, the immunological response may involve the formation both of fixed and of circulating antibodies to various degrees, depending on conditions of immunization. It is the latter type to which the evidence relates, and these may well fluctuate as tumors develop. Probably the cell-fixed antibodies are more likely to be involved in most other pathological aspects of the disease. This opinion derives from analogy with the destruction of homografts, which studies (*cf.* Medawar, 1959) of transplantation immunity show to be primarily a cell-mediated response. In this connection, hypogammaglobulinemia has been observed (Hudson and Wilson, 1960) in neoplastic disease as well as in tuberculosis (Parkes, 1958), the development of delayed hypersensitivity evidently being independent of the production of humoral antibodies.

Transplantation disease is also considered to be due mainly to the activity of cell-fixed antibodies, as in the reciprocal situation of graft destruction by the host. Here, too, the occurrence of circulating antibodies has been reported. Simonsen (1957) found that in the anemia that develops in chicks with transplantation disease, the red blood cells are "Coombs'-positive." In mice showing transplantation disease, Trentin (1958a) reported anemia due to hemolysins, and Porter (1960a, b) and Piomelli and Brooke (1960) reported similar findings for irradiated rabbits that received homologous bone marrow and for nonirradiated rabbits that had received the foreign cells at a late fetal stage.

Auto-Antibodies

The literature on the immunology of cancer (*see* reviews by Hauschka, 1952; Goer, 1956; Zilber, 1958; Barrett, 1958, and Southam, 1960) contains many examples of the occurrence, in the serum of tumor-bearing organisms, of "auto-antibodies" that react with specific constituents of the tumor. For example, it has been reported that extracts of autologous tumors give complement fixation with serum (Graham and Graham, 1955) and positive skin tests (Grace and Dao, 1958). It is possible to regard many of these in reverse manner such that the antibody (in the sense of something new produced by the organism) is represented by the tumor constituent and the antigen by the factor in the serum. It is also possible to regard this situation as a reflection of the normal occurrence in organisms of systems of mutually complementary, specifically interacting, substances as was originally proposed in a "Natural Auto-Antibody Concept" (Tyler, 1940, 1947) and as has since been embodied in template views of the

synthesis of large molecular substances. Many examples of such naturally occurring, interacting substances derived from the same cell, or the same organism, have been previously cited (Tyler, 1940, 1947, 1955, 1956, 1957). The necrosis that occurs in tumors may result in increased liberation of one or both members of such pairs of mutually complementary substances, and this might not be readily distinguished from an immune response of the tumor against "host" antigens. It is also possible that there is no fundamental distinction, if an elective theory of antibody formation, such as proposed by Lederberg (1959a), Talmage (1959), or Burnet (1959), should prove valid (*cf.* Campbell and Garvey, 1960, for a current critique of theories of antibody formation).

Reversibility of Differentiation

Further discussion of antibody formation by tumors raises other questions that are still largely unanswered concerning the kinds of cells that are potentially immunologically competent and concerning the kinds of cells from which various tumors arise. With regard to the latter question, the favored view is of origin from undifferentiated cells normally in the various tissues, but the possibility has not been excluded that tumors may also arise from typical tissue cells that dedifferentiate more or less completely.

Similarly there is uncertainty concerning immunological competence. It is known that cells of the lymphoid series are immunologically competent, and that these are widely distributed throughout the various tissues and organs. Most immunologists attribute the capacity of organisms to exhibit local sensitization to the immunological responsiveness of these cells in the various tissues. Even when present in relatively low proportion, an immunological reaction on their part could, by some mediator such as histamine, account for reaction of nonsensitized tissue cells. The questions of whether or not other kinds of tissue cells can react immunologically, or can transform under various conditions into immunologically competent cells, have not as yet been resolved. Although the evidence has favored the view that this property is restricted to certain types of cells, currently there are indications (*cf.* Rappaport, 1960) that it may be more generally distributed. These are part of the general question of the extent to which cells may dedifferentiate and give rise to new cell types (*cf.* Trinkaus, 1956). For the initial formulation of the present thesis it is not essential that this question be answered. The tumor is considered to arise simply from cells that are immunologically competent or potentially so, regardless of whether they represent a fraction or all of the cells of the tissue.

According to Burnet's (1959) clonal selection theory, cells that are incipient producers of antibodies against an antigen present at the time that they would become immunologically competent (*e.g.*, in the embryo or newborn) are destroyed or inactivated by the antigen as they arise. If this theory were to prove valid in its present form, one could not assume that any kind of cell in which the postulated antigen-deficiency

occurred would be able to develop immunological competence. One would then consider only those cells that already possess this quality to be the source of the tumor. Again it may be emphasized that this question is unsettled, and the possibility remains that also otherwise differentiated cells may acquire immunological competence and, if they have lost certain "host" antigens, can form the corresponding antibodies.

The relation of antibody production to the pathology of cancer may be further illustrated by experiments of Berenbaum (1960) which show that many of the substances used clinically in the treatment of human cancer cases can inhibit antibody formation significantly.

STIMULUS TO PROLIFERATION

Proliferative Response of Tumor Cells

The thesis that tumor cells are immunologically competent places their proliferative property on the same basis as that of antibody-forming cells in general. It now appears, particularly from the work of Coons (1958, 1959; *cf.* Burnet, 1959; Nossal, 1959) that antibody-forming cells receive a specific proliferative stimulus by exposure to foreign antigen. On the basis of the assumption that tumor cells lack one or more "host" antigens, whereby the latter have become foreign to it, these cells are chronically exposed to "foreign" antigen. They would then be expected to proliferate indefinitely, though the rate may fluctuate with conditions that may alter contact with the "foreign" antigen in its effective form (soluble or particulate?) and concentration, and with conditions that affect immunological responsiveness (*e.g.*, irradiation, cortisone, etc.).

Presumably the relevant antigen would not disappear from the precancer cell immediately upon loss or inactivation of the corresponding gene, but would be lost at a rate that is determined by the rate of turnover of such substances in the particular cell type. If the cells are mitotically active, then the antigen would also be diluted out by virtue of distribution to the new cells. On the latter basis, it is understandable that tumors arise or can be induced more readily, the greater the ordinary mitotic activity of the tissue. Hormonal influences, relation of tumor type to age of animal, etc., can be interpreted in these terms. Once the ordinary mitotic activity plus turnover of materials of the cells have proceeded sufficiently far in the descendants of the mutated cells, the now foreign antigen of the "host" stimulates them to continued division. In cells that are not ordinarily mitotically active, antigenic loss would proceed much more slowly, but once attained, a continuing proliferative stimulus would be provided by the "foreign" antigen.

Proliferative Response in Transplantation Disease

The stimulating effect of exposure to foreign antigen on cell proliferation has been noted in several experiments in which parental cells

are injected into the F_1 hybrid. This is well exemplified in experiments on mice by Boyse (1959) and Gorer and Boyse (1959), in which donors of the transplanted cells had been preimmunized with sheep or human red blood cells. Antibody production by these cells served then as a measure of their proliferation in the new host. This showed a rising titer until death of the host, when parental cells were injected into the F_1, whereas in isologous transplants there was a low, persistent output of agglutinin. In homotransplants the antibody production by the donor cells disappeared within 3 days, as would be expected on the basis of the graft-rejection reaction. It is of special interest that the stimulating effect is obtained with one of the parent-hybrid combinations used (strain A splenic cells into A $\times$ C57BL) but not with the other (strain C57BL splenic cells into A $\times$ C57BL). Also in the latter combination, transplantation disease does not occur. It is suggested by Gorer and Boyse (1959) that the C57BL cells are more sensitive to foreign antigen than are the A cells and are thus destroyed by an allergic response to excess antigen in their theoretically tolerant hybrid host.

Another example of a proliferative stimulus provided by foreign antigens appears in some experiments by Feldman and Yaffe (1959). Treatment of irradiated mice with isologous splenic cells conferred no complete protection on the recipient. If the animals were early challenged with a foreign tumor, they all survived indefinitely beyond 210 days. This is attributed to the more effective repopulation of the hematopoietic tissues of the irradiated recipient by virtue of increased multiplication of the donor (isologous) splenic cells in response to the foreign antigen.

A proliferative stimulus to homologous donor cells is also indicated in experiments of Simonsen (1957) showing splenomegaly in chicks; in experiments of Porter and Murray (1958) showing hyperplastic bone marrow in irradiated rabbits, and in experiments by Boyer (1960) which show splenomegaly and hepatomegaly upon inoculation of the chorioallantoic membrane of the chick with adult fowl leukocytes.

Initial Number of Cells

It is assumed that a single altered cell can give rise to a tumor. Experimentally it has been shown (Furth and Kahn, 1937; Hauschka and Levan, 1958; Hewitt and Wilson, 1959) that neoplasms can be transmitted with inoculums that contain only one or two cells. In transplantation disease a large number of cells is generally used, splenic cells being more effective than bone marrow. The required number is also greater in a nonirradiated than in an irradiated host, presumably because the latter provides more "living quarters" for the injected cells in its depopulated spleen and marrow. Possibly the ability to respond to the proliferative stimulus depends largely on the cells becoming lodged in the appropriate organ. Furthermore there are the possibilities that some dedifferentiation must occur in these cells before they can respond and that many may be inactivated by excess host antigen. Coupled with the supposition that tumor cells

have already received their proliferative stimulus before transplantation and, that the precancer cell normally has its niche in the organism, these factors may well account for the apparent discrepancy in numbers of cells required for tumor initiation and for transplantation disease.

INDICATED DIRECTIONS OF THERAPY

The hypothesis that cancer is initiated by gene loss or inactivation limits somewhat the possible directions from which one might reasonably expect nonsurgical therapeutic measures to be developed.

Supplying DNA or RNA

Conceivably the gene loss might be repaired by supplying the appropriate genetic material to the cells as has been done in genetic transformation by deoxyribonucleic acid (DNA) in pneumococci and other microorganisms. However, the efficiency of this phenomenon is very low, though it has been raised to as high as 17 percent of the cells under special conditions (Hotchkiss, 1955). Possibly the normal DNA might be conjugated to a carrier virus to create a transducing particle (*cf.* Lederberg, 1959b) that might bring the efficiency to the necessary 100 percent. The DNA could presumably be derived from autologous or isologous normal tissues (*e.g.*, spermatozoa, liver, etc.) directly or after cultivation *in vitro*. At present these possibilities seem remote. If gene inactivation rather than loss is assumed, there are at present no real clues as to methods for reversing this process in 100 percent of the cells.

In accordance with current concepts, it may be assumed that the determination of the specificity of cellular antigens by the DNA of the genes is mediated by ribonucleic acid (RNA). It should, then, be theoretically possible to restore the missing antigen by use of the appropriate RNA. An experiment of this type has actually been done recently by M. C. Niu (1960) who reports in a brief abstract that the transplantability of ascites tumor cells of mice could be greatly suppressed (10% instead of 96% "takes") by preincubation with RNA derived from calf liver, but not by tumor RNA nor yeast RNA. That *calf* RNA should be able to "normalize" *mouse* tumor cells does seem rather surprising. It is not quite in accord with the present thesis unless, somehow, the specificity of the missing mouse cell antigen is supplied by the calf RNA.

Radiation Treatment

Theoretically it should be possible to induce further gene loss or inactivation by exposure of tumors to low doses of ionizing radiations or to radiomimetic substances. In such case the altered cell(s) would be tolerated by the tumor and would react immunologically against the

tumor. Some tumor destruction could thus be achieved while the altered cells were developing into a new tumor.

Intensive irradiation for the purpose of destroying all the neoplastic cells would be expected to affect also normal host cells and replace one neoplasm by another, with some time gained by virtue of the latent period. Whole-body irradiation at supralethal doses combined with injection of isologous bone marrow has been used by Barnes *et al.* (1959) with some success in prolonging the life of mice with leukemia (*see*, however, Hewitt and Wilson, 1959). Presumably most of the mitotically active normal tissue, as well as the tumor tissue is destroyed by this treatment. Gene loss in the nondividing cells would not manifest itself in the form of a tumor for a considerable time. For use with noninbred animals, such as man, the replacement of destroyed hematopoietic tissue in this procedure would have to be achieved by use of autologous cells (collected prior to irradiation) or of a type of homologous cell (*e.g.*, fetal liver cells) that would be least likely to cause transplantation disease (Barnes and Loutit, 1959; Vos *et al.*, 1960).

Passive or Active Immunization

This subject has been extensively reviewed (Hauschka, 1952; Gorer, 1956; Zilber, 1958; Barrett, 1958; Southam, 1960). It is well known that antibodies produced in another animal against tumor cells, or extracts therefrom, can damage or destroy these cells *in vitro*. However, there has not been any clear-cut demonstration that the passive administration of antiserums to a tumor-bearing animal would affect only the neoplastic cells. According to the present thesis the tumor cells have lost one or more antigens, but still possess others characteristic of the parent cells. One would not, then, expect to obtain a selectively acting antiserum by the procedure of absorbing an antitumor antiserum with other tissue materials.

Conceivably the loss of a particular antigen may permit the expression of some other antigen not ordinarily found in the cells of the body. While current evidence is against this, the possibility should not be neglected. To be susceptible to the action of antibodies it is to be expected that such antigens would be part of the surface of the cell. Experiments with normal dividing fertilized egg cells of sea urchins (Tyler and Brookbank, 1956; Tyler, 1957, 1959) have shown that a purified mucopolysaccharidic constituent of the surface of the cell can act as an immunizing antigen and induce the formation of effective cell-division-blocking antibodies.

The possibility of using tumor extracts as autologous vaccines has been explored by a number of investigators, and recently Graham and Graham (1959) report favorable effects in humans. The procedure involves the use of Freund's adjuvant, which has been found to be highly effective in enhancing the immunizing ability of homologous and heterologous antigens and in enabling autologous antigens, such as materials of nerve, testis, or thyroid, to induce a response that damages these organs (*cf.*

Freund, 1956; Voisin, 1958; Waksman, 1959). While there has been some doubt about the immunological nature of the effect of autologous materials, this seems to be largely removed by recent experiments, such as those of Paterson (1960) who reports successful transfer of allergic encephalomyelitis by means of lymph-node cells. It has been suggested that the ability to act auto-antigenically is related either to isolation of the particular tissue from the circulation or to its development after the organism becomes immunologically competent. Whether this view will be found valid and whether tumor tissue will be found to be in this category needs yet to be experimentally determined.

Allergic Injury to Cells

If the pathological manifestations of cancer are due to the immunological activity of the tumor cells, then treatment can be based on principles derived from studies of allergic reactions. Thus, agents that would interfere with antibody formation (*e.g.*, cortisone, X irradiation) or with secondary effects of antigen-antibody reaction (*e.g.*, antihistaminics) may be expected to provide temporary relief or remission, as they are known to do. In addition, one may envisage complete destruction of the tumor cells by exposure to high concentrations of the relevant antigen.

Tissue-culture experiments by Rich and Lewis (1932) and many other investigators (*cf.* Rich, 1951; Favour, 1957) have shown that cells from a sensitized animal can be damaged or destroyed by exposure to sufficient concentration of the sensitizing antigen. The stimulus to proliferation, to which reference has been made, is obtained at lower concentrations and under other conditions, as illustrated in the experiments of Waksman and Matoltsy (1958). If we assume that tumor cells are sensitized to host antigen, but normally are exposed to low concentrations of the relevant antigen, it should be possible to destroy them by administration of a large enough dose. Since the tumor represents a relatively small fraction of the tissue mass of the body, it is possible that this may be accomplished without fatal anaphylactic reaction. The problem should be similar to, but perhaps simpler than, that of desensitization in allergies of the delayed type. For the latter there have been reports (*e.g.*, Uhr, 1958) of the achievement of complete desensitization in experimental "allergic" animals by use of relatively large amounts of antigen. Also in experimental encephalomyelitis it has been reported (Shaw *et al.*, 1960) that the induction of this condition by injection of whole-brain extract in Freund's adjuvant can be suppressed by intradermal injection of homologous neural proteins.

Some recent experiments by Druckrey *et al.* (1959) provide evidence that extracts of normal tissues may have a destructive action on tumor cells. They found that homogenates of spleen, lung, and muscle could cytolyze tumor cells of the rat *in vitro*. Except for red blood cells, most normal tissue cells were not damaged by the homogenates. The material proved highly toxic, however, to the whole organism.

Examples of cell suppression by excess antigen are available also from experiments on transplantation disease. Thus Cosgrove *et al.* (1959) and Upton *et al.* (1959) were able to inhibit this reaction on the part of irradiated mice receiving foreign splenic cells, by a preincubation of the donor cells with recipient-strain liver cells. The inhibition was not obtained if donor-liver cells were used. They interpret this inhibition as an effect of exposure of the donor cells to too high a concentration of prospective host antigen.

The application of this principle of administration of excess antigen in human cancer would presumably be best done by use of autologous material. This could conceivably be derived from some normal tissue, directly or after cultivation, or from some body secretion such as the saliva. In instances where the antigens lost by the tumor cells represented one of the blood-group antigens, purified materials (*cf.* Kabat, 1956) would be available from this or related sources. The antigens could also be derived from homologous sources, though response of the tumor, as well as of antibody-forming cells of the "host" to other foreign antigens, might well interfere with the desired effect.

In summary, then, the present analysis does indicate some directions from which therapeutic measures may be developed and suggests experimental tests of their feasibility and possible effectiveness.

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Lung Cancer in World War I Veterans: Possible Relation to Mustard-Gas Injury and 1918 Influenza Epidemic [1, 2]

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SUMMARY

Army and Veterans Administration records were used to investigate the possible significance of influenza and mustard-gas poisoning in the etiology of lung cancer. Three groups of World War I Army veterans were traced for mortality from 1919 through 1955: 1,855 men who had pneumonia during the influenza pandemic, 2,718 men who were hospitalized for mustard-gas poisoning in 1918, and a control group of 2,578 soldiers who had wounds of the extremities. No relationship was seen between pneumonia in 1918 and subsequent cancer; there was, however, suggestive evidence that the incidence of lung cancer was slightly increased in men who had been subjected to mustard-gas poisoning in 1918.—J. Nat. Cancer Inst. 25: 1231–1252, 1960.

IN THE 1920 report (*1*) of their pathological studies of the influenza epidemic in the United States, Winternitz and coworkers commented on the striking similarity of the epithelial proliferation seen in some fatal cases of postinfluenzal pneumonia and in an invasive, malignant neoplasm. These findings were so dramatic the investigators were led to speculate that the influenza pandemic of 1918–19 might later lead to some increase in the incidence of respiratory cancer. Laboratory experiments with influenza virus in mice (*2–4*) have not produced support for this hypothesis. The current emphasis in cancer research is on specific carcinogens, an emphasis greatly strengthened by the results of epidemiological studies on the smoking of tobacco, but there is also historical evidence, recently reviewed by Finke (*5*), which suggests that virtually any chronic pulmonary lesion may enhance the risk of lung cancer. Chronic bronchitis has been shown in the 1951 Impairment Study of the Society of Actuaries (*6*) and in a report by Case and Lea (*7*) to imply

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increased risk of lung cancer, and Papanicolaou (*8*) lent some support to the view that a protracted chronic inflammatory process may pave the way for the development of a malignant respiratory neoplasm.

A further stimulation for the present study derives in part from experimental work on the carcinogenic activity of both nitrogen and sulfur mustards (*9–12*) in mice of a strain known to be highly susceptible to the development of pulmonary tumors. In addition, occasional isolated cases of lung cancer in man have been reported after occupational exposure to mustard compounds (*13*). In contradistinction to the experimental work on the influenza virus as a carcinogen, that on the mustards is quite provocative, so that any really relevant human data would be of great interest.

After the present study had been planned, the report of Case and Lea (*7*) appeared and seemed to provide a partial answer to the question about mustard gas. In a sample of 1,267 British World War I veterans alive on January 1, 1930, and receiving pensions for the effects of mustard-gas poisoning in 1917–18, they found 29 deaths from cancer of the lung and pleura in contrast to 14 expected on the basis of mortality rates for the male population of England and Wales over the calendar period of observation; in addition, there were 217 deaths from bronchitis versus 21 expected. Since the pensions usually had been granted because of chronic bronchitis, Case and Lea added a parallel series of 1,421 men pensioned for bronchitis but who had not been exposed to mustard gas. In this series also 29 deaths were observed from cancer of the lung and pleura versus 14 expected. Since the the same discrepancy as to lung cancer was found in the two series, Case and Lea concluded that mustard gas had not acted as a direct carcinogen, but had increased the risk of lung cancer indirectly through the mechanism of bronchitis. In a control group of 1,114 amputees studied in the same way, they found 13 deaths from lung cancer in comparison with 15.5 expected.

MATERIALS AND METHODS

Epidemiologic and follow-up studies on the veteran population (*14–18*) have demonstrated that this large segment of the U.S. male population is readily sampled and highly accessible to follow-up study. Medical record systems of the Armed Forces and the Veterans Administration are standardized, usually include punch-card indexes, and provide for central filing and access to all individual records. It appeared, therefore, that both problems involving lung cancer could be efficiently investigated via U.S. Army World War I veterans chosen on the basis of history in 1918.

The nature of the original observations by Winternitz *et al.* (*1*) on fatal cases of pneumonia implied that the population of interest was not the 792,000 U.S. Army primary admissions for influenza in World War I but the estimated 100,000 secondary cases (*19*, *20*) of pneumonia plus 78,000 primary cases of pneumonia. The pandemic consisted of a number of

waves during the period 1917–20, but its peak was reached in October, 1918. Accordingly, admissions were sampled from daily reports prepared by the numbered base hospitals in the American Expeditionary Forces which showed name, serial number, unit, and diagnosis. The period used was September through November, 1918.

Sulfur mustard was a war gas in World War I and led to an estimated 28,000 casualties in the U.S. Army (*21*). Admissions for gas poisoning in the A.E.F. were first recorded in February, 1918. However, it was not until the summer of 1918 that the daily hospital "report of casualties and changes" routinely showed the probable nature of the gas, so that it was necessary to sample admissions from August through October, 1918, and to depend chiefly on the special gas hospitals (*21*).

The most important problem visualized in sampling the mustard-gas injuries was the validity of the diagnosis. It was felt necessary to minimize the two chief errors of diagnosis: (1) influenza or other respiratory disease wrongly diagnosed as mustard-gas injury, and (2) the man injured by another gas whose record alleges mustard gas to be the agent. Accordingly, any case was rejected unless either the skin or the eyes were affected. If the clinical records were equivocal in attributing the injury to mustard gas, it was required that there be evidence of burns of the skin. In addition, it was required for all mustard-gas cases that there be evidence of some injury to the respiratory tract.

Although a reasonable basis of expected mortality in the present study might be provided by appropriate general mortality rates, it was considered safer to include an explicit control group for which mortality information would be obtained and processed in the same way as for the pneumonia and mustard-gas groups. Men with wounds of the extremities were selected as the most nearly comparable diagnostic group.[4]

Men born in the years 1888 through 1893 were chosen. The sample was further restricted to white males alive on January 1, 1919.

To determine sample size, preliminary computations were made on the U.S. life table for white males in 1930 and on the data of Cutler and Loveland (*22*), which suggested that about 35 percent of the men on the proposed rosters would have died from all causes by the end of 1956, and approximately 1 percent from lung cancer. Calculation showed that 2 samples of 2,500 each, in tests based on the 0.01 criterion of significance, would have about 60 percent power (in the Neyman-Pearson sense) in detecting a differential lung-cancer mortality of 2 to 1, and 99 percent power against a differential of 3 to 1, if expectation were 1 percent in the controls. Accordingly, the desirable sample size was set at 2,500 for each of the 3 rosters. The various restrictions, however, led to a sample of 1,855 men in the pneumonia group, 2,718 men in the mustard-gas group, and 2,578 in the control group with wounds of extremities.

[4] Case and Lea (*7*) make a point of the saturation of areas of French soil with mustard gas and stress the desirability of selecting controls from among men who had never served in France after July 12, 1917, when mustard gas was first used. The necessity for this restriction had not been suggested by review of the U.S. Army historical sources (*21*), and it was deemed sufficient to examine in detail the medical history of each potential control to insure that he had never been gassed, regardless of type of gas.

NATURE OF OBSERVATIONS

Baseline Data From Military Records

In addition to such information as date and place of birth, date of enlistment, and military unit, clinical records were abstracted with respect to date of admission for reference illness, supporting evidence of gas injury (involvement of eyes), supporting evidence of mustard-gas injury (involvement of skin), evidence of injury to respiratory tract, and complicating history.

The 3 rosters proved to be quite homogeneous with respect to place and year of birth. However, while the mustard-gas and control rosters were drawn overwhelmingly from the infantry (79 and 88%), only 52 percent of the pneumonia cases were infantrymen.

Mortality Data

Records of substantially all deaths of World War I veterans are in VA central files. The latter were therefore used to determine the mortality experience of veterans selected for this study. Initial processing established the fact of death or survival on January 1, 1956, and for all 2,542 veterans known to have died the VA claims folders were reviewed. Deaths before January 1, 1956, numbered 2,441, and these are used in the subsequent analysis. Proof of death is contained in these folders, ordinarily as a photostatic copy of the death certificate. If cancer appeared on the death certificate, a copy of the autopsy protocol, if any, was requested. If cancer did not appear on the death certificate, the responsible medical authority was asked whether cancer was an autopsy finding. If cancer was so reported, then a copy of the protocol was obtained.

By these procedures comparable mortality information was obtained on the several rosters, both at the level of the death certificate and the postmortem examination. The 3 rosters compared very closely with respect to the relative frequency of autopsy, 18.6 percent in the pneumonia roster, 20.7 percent in the mustard-gas roster, and 19.3 percent in the controls. For about a third of the deaths in each roster it was clear that no autopsy had been done, and for nearly half it was unknown whether autopsy had been done.

Of all 76 deaths during 1919–55 with primary cause cancer of the trachea, bronchus, and lung, 33 are known to have been brought to autopsy, 20 were stated not to have been so studied, and in 23 it is uncertain whether autopsy was performed. The proportions do not vary appreciably by roster. Review of the 31 autopsy protocols which could be obtained showed that lung cancer was found as primary site in 27 and as secondary site in 3. In but a single case autopsy failed to confirm respiratory cancer, stated on the death certificate to be the primary cause. In this case the right lung had previously been removed for squamous-cell carcinoma. Histologic information was sought, but since only

27 protocols were obtained for primary lung cancer the results were unrewarding.

In addition, among the 354 autopsy protocols obtained for deaths in which the death certificate gave a primary cause other than lung cancer, 5 contained evidence of lung cancer: 2 in the mustard-gas roster, 1 in the pneumonia, and 2 in the controls. It was concluded that the death certificate diagnoses were unbiased with respect to the 3 rosters, and accurate enough to serve as the basis for the roster comparisons to which the study was directed.

For purposes of analysis each death was coded as to cause. The primary or underlying cause of death was determined on the basis of the death-certificate together with coding rules developed for use with the last two revisions of the *International Statistical Classification of Diseases, Injuries and Causes of Death*. The assistance of the National Office of Vital Statistics proved invaluable. The rubrics of the Sixth Revision were employed for coding purposes. In addition to the primary cause, other causes or related conditions stated on the death certificate were also coded and without further attempt to determine priorities among them.

Morbidity and Disability Data

The study was enlarged to include morbidity after Case and Lea had suggested that an excess of lung-cancer deaths among victims of mustard-gas injury in World War I might arise via the intermediation of chronic bronchitis. As a practical matter, only illnesses that led to hospitalization within the VA system could be systematically brought into the study. There is reason to expect VA hospital reporting to provide a biased roster comparison, since veterans with gas injury in World War I would be expected to apply for VA hospitalization more often than other veterans with bronchitis. However, interest in chronic bronchitis arose only out of the possibility that bronchitis might be a factor in any excess lung-cancer mortality that the mustard-gas or pneumonia rosters might show. This possibility can be examined by comparison, within a particular roster, of the probability of a history of bronchitis for veterans who died of lung cancer with that for men who did not. If the suggestion of Case and Lea held for the American veterans, this would be reflected by an excess of bronchitis among men who died of lung cancer. Information was therefore obtained for a 10 percent sample of all men alive on January 1, 1956, or who died prior to that date of causes other than lung cancer, and for all men who died of lung cancer before that date. Note was made of VA hospitalizations for diseases of lung, bronchi, and pleura and of diagnoses made on an outpatient status, as in connection with the adjudication of claims. Note also was made of all diagnoses acknowledged to exist at the last VA examination for rating purposes, regardless of service connection.

Smoking History

Through the generosity of Dr. Harold F. Dorn, of the National Institutes of Health, it became possible to inquire into the smoking history of a

small subsample of each roster to learn whether any of the roster conditions had affected the smoking history. Dr. Dorn made available his file of questionnaires about smoking that were returned by World War I veterans with VA insurance in force on December 31, 1953. Collation of the files on the 2 studies yielded 170 men covered by both, 66 from the mustard-gas roster, 36 from the pneumonia roster, and 68 from the control roster. For all 170 men, only 34, or exactly 20 percent, declared that they had never used tobacco in any form; 52, or 31 percent, claimed never to have used cigarettes. Among the 118 who were at one time cigarette smokers, 35, or 30 percent, declared they had stopped before returning Dr. Dorn's questionnaire.

The percentages of men claiming never to have smoked do not vary significantly, being 18 for the mustard-gas group, 31 for the pneumonia roster, and 16 for the control group. Similarly, those alleging never to have smoked cigarettes also vary insignificantly, being 27, 42, and 28 in the same sequence. The percentage who stopped smoking cigarettes varies somewhat more, but also insignificantly (P about 0.11), being 40, 33, and 20 in the same order. The percentage who stopped before age 40, however, does vary significantly (P about 0.01), being 25, 14, and 4 in the same order. That is, men who were gassed almost certainly cut down on their smoking. The change in smoking habits thus implied might well reduce the incidence of lung cancer among men who were gassed.

FINDINGS AS TO MORTALITY

Mortality From All Causes

Table 1 shows the comparison of observed and expected numbers of deaths for each of the 3 rosters in various time intervals from 1919 through 1955 as well as for the entire period. The expected numbers of deaths were calculated on the basis of death rates for the U.S. white male population over the calendar period of interest. For this purpose U.S. life tables for white males in 1919–21, 1929–31, 1939–41, 1949–51, and 1955 were drawn upon for age-specific mortality rates in decennial census years and 1955, the rates for intervening years being obtained by linear interpolation. This made possible the determination of net mortality and survival ratios for any particular year-of-birth cohort.

Comparison of 1919–55 total figures indicates that the 3 rosters differ among themselves, and that in all of them mortality was either somewhat under-reported or actually below that of U.S. white males. In table 1 the experience is divided into broad time intervals, from which it may be seen that the deficit in observed mortality is largely confined to the first decade of the study period, when the effect of military screening would have been most influential. This comparison suggests that the mortality experience of World War I veterans has been somewhat more favorable than that of U.S. white males generally.

TABLE 1.—Observed and expected deaths, by roster, in relation to calendar time

Calendar period	Number of deaths		Ratio
	Observed	Expected	Observed/Expected
Mustard-gas roster			
1919–29	116	163	0. 71
1930–39	248	190	1. 31
1940–49	312	323	0. 97
1950–55	276	298	0. 93
Total	952	974	0. 98
Pneumonia roster			
1919–29	94	112	0. 84
1930–39	121	130	0. 93
1940–49	222	221	1. 00
1950–55	206	203	1. 01
Total	643	666	0. 97
Control roster (wounded)			
1919–29	95	155	0. 61
1930–39	167	180	0. 93
1940–49	300	307	0. 98
1950–55	284	283	1. 00
Total	846	925	0. 91

Text-figure 1 presents observed death rates for each roster and for U.S. white males of comparable age, and reveals that:

1) In the first postwar decade mortality in all 3 rosters was well below that for U.S. white males of comparable age, but among men on the pneumonia roster the rate was rather higher than among men on the other two rosters.
2) In the second decade the death rate for men on the mustard-gas roster advanced rapidly to occupy a position well above that for the other two rosters and for U.S. white males; the latter three curves were very close.
3) In the third decade the several rosters were indistinguishable from one another and from U.S. white males.

Mortality by Cause

Primary interest here, of course, attaches to death from lung cancer and other conditions involved in the hypotheses underlying the investigation. The latter include, for mustard gas, neoplasms of lymphatic and hematopoietic tissues (especially leukemia), anemias, and respiratory diseases generally. For pneumonia the conditions of interest, in addition to lung cancer, are respiratory diseases generally. However, in addition, the whole spectrum of cause was reviewed.

Lung Cancer in Relation to Roster Conditions

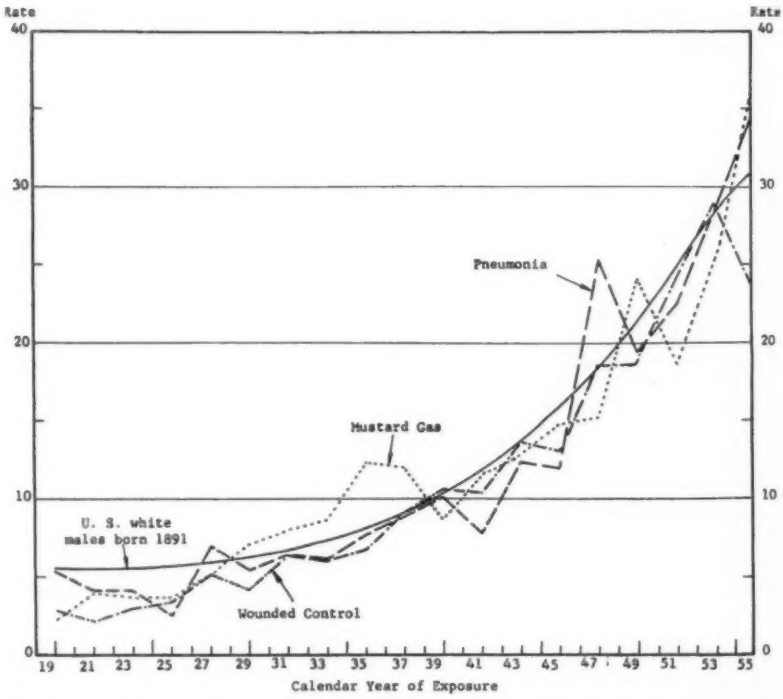
The precise rubrics of interest are the following in the Sixth Revision of the International Lists:

162 Malignant neoplasm of trachea, and of bronchus and lung, specified as primary.

163 Malignant neoplasm of lung and bronchus, unspecified as to whether primary or secondary.

For the period 1919–55, there were 76 deaths so classified as to primary cause; the number of deaths in which these diagnoses appear as associated conditions is negligibly small, 1 in the mustard-gas and 1 in the control roster. Moreover, such autopsy evidence as could be obtained failed to suggest that many deaths from lung cancer were actually attributed to other conditions. Lung cancer was found in 5 among 354 postmortem examinations on men whose deaths were ascribed to conditions other than lung cancer. These are distributed among the 3 rosters, and in only one was another pulmonary disease (bronchial pneumonia) given as primary cause of death. In one instance an early squamous-cell carcinoma of the bronchus was an incidental finding in a death attributed to portal cirrhosis, while in 3 cases the death certificate gave as primary cause a malignancy of another site shown on autopsy to be metastatic extension of bronchogenic carcinoma.

In the absence of competent autopsy of every death, one cannot have an entirely satisfactory count of deaths either from lung cancer or in which lung cancer is demonstrable. The available counts clearly include



TEXT-FIGURE 1.—Deaths per 1,000 men per year, by calendar period of exposure, and by roster, in comparison with U.S. white males of comparable age.

few deaths attributed to lung cancer in which this condition did not exist; among 31 autopsies none falls into this category, although in 3 the lung was found to be a secondary site. While errors of exclusion are undoubtedly present, there is no suggestion or reason to believe that the roster comparisons are in any way biased by such errors.

The design of the present study assumes a direct comparison of two independent samples, and when this method of comparing the rosters is used, the ratios of lung-cancer deaths to total sample size become:

For mustard-gas vs. wounded controls

36/2,718 vs. 26/2,578
or
1.3 percent vs. 1.0 percent
for which a one-tailed P is about 0.17.

For pneumonia vs. wounded controls

14/1,855 vs. 26/2,578
or
0.8 percent vs. 1.0 percent, which does not suggest a positive association of lung cancer with pneumonia.

These comparisons may be made directly and simply because the samples are homogeneous as to age. In both instances, therefore, the conclusion is clear that the observed discrepancies lie well within the usual range of sampling variation.

The sampling plan was devised so that the study would have a high chance (about 99%) of detecting a differential of 3 to 1, and a 60 percent chance of detecting a differential of 2 to 1, with 0.01 employed as the significance level. The evidence since published by Case and Lea suggests that a 2 : 1 ratio might be observed for the mustard-gas versus wounded comparison.

Although it was not planned that the analysis of the present study should rest on expected values calculated from the experience of the general population, in view of Case and Lea's results it seems essential to make such calculations for the 3 study rosters. For this purpose, rates for all cancer of the respiratory system have been used rather than for lung cancer. For U.S. white males the National Office of Vital Statistics provided annual age-specific rates for 5-year age groups, from which rates for each single year of age were obtained by interpolation. These were applied to the observed years of exposure at each age in the interval 1919–55 to obtain the expected number of deaths from cancer of the respiratory system. On this basis 71.4 deaths would have been expected in the entire sample in contrast to 84 observed, a statistically insignificant discrepancy. Table 2 presents these observed and expected deaths over the range of age at exposure, and by roster.

When the roster comparison is approached on the basis of expected values calculated from death rates for U.S. white males, rather than by a direct comparison of rosters processed in identical fashion, the results are

TABLE 2.—Observed and expected deaths attributed to malignant neoplasms of respiratory system, by age at death and by roster

Age at death	Mustard-gas		Pneumonia		Wounded controls		Total	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
26–29	0	0. 03	1	0. 02	0	0. 03	1	0. 08
30–34	0	0. 13	0	0. 09	0	0. 12	0	0. 34
35–39	0	0. 27	0	0. 19	0	0. 26	0	0. 72
40–44	1	0. 66	0	0. 45	0	0. 64	1	1. 75
45–49	5	1. 85	0	1. 28	2	1. 80	7	4. 93
50–54	3	3. 80	5	2. 66	1	3. 73	9	10. 19
55–59	13	7. 69	5	5. 36	12	7. 55	30	20. 60
60–64	13	10. 17	3	7. 09	12	10. 07	28	27. 33
65–67	4	2. 04	1	1. 42	3	2. 01	8	5. 47
Total	39	26. 64	15	18. 56	30	26. 21	84	71. 41

quite different. A summary for all cancer deaths of the respiratory system is as follows:

Roster	Observed deaths	Expected deaths	P*
Mustard-gas	39	26. 6	<0.01
Pneumonia	15	18. 6	>0.05
Controls	30	26. 2	>0.05

*One-tailed for mustard-gas and pneumonia, two-tailed for controls.

Since a significant proportion of the men on the mustard-gas roster had given up cigarette smoking prior to age 40, it may be asked whether this might have served to reduce an otherwise greater excess of lung cancer in these men. The sample of men with smoking histories—only 170—is much too small to permit a direct correction for this possibility, but an upper bound can be set on the possible error. This can be done by making the extreme assumption that men who stopped smoking cigarettes before age 40 were subject to the same risk of lung cancer as were nonsmokers. It is estimated that 15 percent of the men on the mustard-gas roster were at one time regular cigarette smokers, but gave up smoking as a result of being gassed, while the corresponding percentage for the pneumonia roster is 6 percent. The percentage of regular cigarette smokers in each roster at the time of Dorn's survey was 44 for the former and 39 percent for the latter. On another extreme assumption, *viz.*, that *all* lung cancer occurred in regular cigarette smokers, then the addition of 15 to the 44 percent cigarette smokers in the lung-cancer roster might be expected to increase the number of deaths from lung cancer by about a third. For the pneumonia roster, the similarly derived upper limit would be an increase of about one sixth in the number of deaths from lung cancer.

Since the effect of increasing the 1.3 percent incidence of lung cancer in the mustard-gas roster by a third would be to achieve a level of about 1.7 percent, it appears that, even had there been no alterations of smoking patterns, the lung-cancer rate in the mustard-gas group would not achieve

twice control levels. With the pneumonia roster the effect would be smaller and the "adjusted" percentage with lung cancer only 0.9.

As the present study was originally designed it fails to prove, beyond reasonable doubt, that mustard-gas injury in World War I had a carcinogenic effect. However, the observations are not inconsistent with those of Case and Lea, who reported a 2:1 ratio of observed-to-expected deaths in British veterans drawing war pensions for chronic bronchitis after mustard-gas injury in World War I. Although the analysis of Case and Lea rests on the indirect comparison of rosters through the medium of death rates in the general population, the applicability of the latter to British veterans in 1930–52 seems well established by the agreement Case and Lea obtained between deaths expected and observed, among men in their control sample, not only for all causes and for lung cancer but also for cancer of other sites. In view of the close agreement in the present study between observed and expected respiratory-cancer deaths for the pneumonia and control rosters (45 vs. 44.8), parallel reasoning might be applied to the present study to favor the conclusion that the observed and expected values for the mustard-gas roster (39 vs. 26.6) constitute a valid comparison. It is concluded from the present study that it is extremely unlikely that the influenza epidemic of 1918 has contributed in any way to the increasing death rate from lung cancer in the United States.

Other Causes of Particular Interest

Since the mustard-gas sample was chosen for acute respiratory involvement, and since the mustards are regarded as radiomimetic, analysis was made of deaths attributed to leukemia and lymphoma, anemias, and diseases of the respiratory system. However, except for diseases of the respiratory system, none of these conditions was very often mentioned as either the principal or the associated cause of death, and there was no evidence of significant roster variations with respect to any of them. Thus, leukemia and aleukemia (code 204) was cited 14 times as cause of death, 5 in each of the mustard-gas and pneumonia rosters, and 4 in the control roster.

Diseases of the respiratory system, exclusive of tuberculosis and neoplasms, accounted for 96 deaths among men on the mustard-gas roster and 50 among men on each of the other 2 rosters. The difference between the mustard-gas and control rosters is well outside the expected range of chance, but that between the penumonia and control rosters is small and of borderline significance (P about 0.06) in a one-tailed test. The percentages dying of respiratory diseases over the interval 1919–55 are 3.5 for the mustard-gas roster, 2.7 for the pneumonia roster, and 1.9 for the control roster. Mortality trends over time are exhibited in table 3 to facilitate comparison of the 3 rosters. Except for the period 1954–55 the respiratory-death rate is highest for the mustard-gas roster, especially in the period 1934–38. From the particularly elevated rates for the mustard-gas roster in the period 1934–38, it would appear that the

TABLE 3.—Number of deaths and deaths per 1,000 men per year for respiratory disease,* by roster, and by period of exposure

Period of exposure	Mustard-gas		Pneumonia		Wounded controls	
	Deaths	Rate	Deaths	Rate	Deaths	Rate
1919–23	10	0. 74	6	0. 66	0	—
1924–28	9	0. 68	4	0. 45	5	0. 40
1929–33	15	1. 17	10	1. 15	10	0. 81
1934–38	24	1. 96	10	1. 18	10	0. 84
1939–43	11	0. 95	5	0. 62	6	0. 53
1944–48	12	1. 10	7	0. 92	9	0. 84
1949–53	11	1. 11	4	0. 58	5	0. 51
1954–55	4	1. 09	4	1. 60	5	1. 40
Total	96	1. 09	50	0. 83	50	0. 59

*Exclusive of tuberculosis and neoplasms.

respiratory diseases contribute heavily to the high general mortality of men on this roster during this period, as noted earlier.

In view of the large excess of deaths from respiratory diseases among men on the mustard-gas roster, the distribution within the individual diagnostic rubrics of this general category is of particular interest, and the significant detail is given in table 4. For no other respiratory diagnosis are deaths frequent enough to warrant analysis.

From the data of table 4 it is apparent that the mustard-gas roster had an excess of deaths from pneumonia but not from the other respiratory diseases. The 1918 pneumonia roster had a relative excess of later deaths from pneumonia of only borderline significance, and no excess from other respiratory diseases.

When the deaths from respiratory disease are distributed over time so as to permit comparisons of trends between pneumonia and other respiratory diseases, additional meaning is given to the temporal variation seen in table 3. As may be seen from table 5, the two respiratory components

TABLE 4.—Comparisons of rosters as to deaths from pneumonia and from other respiratory diseases, 1919–55

Cause of death		Mustard-gas		Pneumonia		Wounded controls	
Code	Title	Deaths	Percent of roster	Deaths	Percent of roster	Deaths	Percent of roster
480	Influenza with pneumonia	9	0. 3	7	0. 4	2	0. 1
490	Lobar pneumonia	37	1. 4	21	1. 1	19	0. 7
491	Bronchopneumonia	23	0. 8	7	0. 4	12	0. 5
493	Pneumonia, other and unqualified	9	0. 3	3	0. 2	3	0. 1
	Subtotal—Pneumonia	78	2. 9	38	2. 0	36	1. 4
480–527	Respiratory disease other than above	18	0. 7	12	0. 6	14	0. 5
	Total	96	3. 5	50	2. 7	50	1. 9
P in one-tailed tests against wounded controls:							
	Pneumonia		<0. 01		0. 06		
	Other diagnoses		>0. 05		>0. 05		

TABLE 5.—Deaths and annual death rates per thousand for pneumonia and for other respiratory diseases, by roster, over time, 1919–55

Period	Mustard-gas roster				Pneumonia roster				Control roster			
	Pneumonia deaths		Other deaths		Pneumonia deaths		Other deaths		Pneumonia deaths		Other deaths	
	Number	Rate	Number	Rate	Number	Rate	Number	Rate	Number	Rate	Number	Rate
1919–28	17	0. 64	2	0. 07	8	0. 44	2	0. 11	4	0. 16	1	0. 04
1929–38	37	1. 47	2	0. 08	18	1. 05	2	0. 12	18	0. 74	2	0. 08
1939–48	16	0. 71	7	0. 31	7	0. 45	5	0. 32	10	0. 45	5	0. 23
1949–55	8	0. 59	7	0. 52	5	0. 53	3	0. 32	4	0. 30	6	0. 45
Total	78	0. 89	18	0. 20	38	0. 63	12	0. 20	36	0. 42	14	0. 16

TABLE 6.—Comparison of study rosters as to mortality from broad groups of causes, 1919–55

Cause of death		Mustard-gas		Pneumonia		Wounded controls		P*
Code	Title	Number	Percent	Number	Percent	Number	Percent	
00	Tuberculosis, respiratory	99	3.6	54	2.9	54	2.1	<0.01
01	Tuberculosis, other	3	0.1	4	0.2	2	0.1	>0.05
02	Syphilis and sequelae	13	0.5	8	0.4	11	0.4	>0.05
04–13	Other infectious and parasitic diseases	8	0.3	6	0.3	8	0.3	>0.05
14	Cancer, mouth and pharynx	7	0.3	2	0.1	7	0.3	>0.05
15	Cancer, digestive system	57	2.1	32	1.7	51	2.0	>0.05
16	Cancer, respiratory system	39	1.4	15	0.8	30	1.2	>0.05
17–19	Cancer, other and unspecified sites	19	0.7	12	0.6	22	0.9	>0.05
20–23	Other neoplasms	16	0.6	15	0.8	18	0.7	>0.05
24–28	Allergic, endocrine, nutritional diseases	13	0.5	12	0.6	18	0.7	>0.05
29	Diseases of blood and blood-forming organs	3	0.1	1	0.1	4	0.2	>0.05
30–32	Mental, psychoneurotic, and personality disorders	11	0.4	4	0.2	11	0.4	>0.05
33–39	Diseases of nervous system and sense organs	44	1.6	52	2.8	57	2.2	0.03
40–41	Rheumatic fever and rheumatic heart disease	12	0.4	9	0.5	8	0.3	>0.05
42	Arteriosclerotic and degenerative heart disease	225	8.3	173	9.3	216	8.4	>0.05
43–46	Other diseases of circulatory system	73	2.7	49	2.6	59	2.3	>0.05
47–52	Diseases of respiratory system	96	3.5	50	2.7	50	1.9	<0.01
53–58	Diseases of digestive system	55	2.0	36	1.9	52	2.0	>0.05
59–61	Diseases of GU system	33	1.2	13	0.7	27	1.0	>0.05
69–79	All other diseases and conditions	15	0.6	7	0.4	16	0.6	>0.05
	Trauma	109	4.0	87	4.7	124	4.8	>0.05
	Unknown	2		2		1		
	Total	952	0.1	643	0.1	846	0.0	
	Number of men	2,718	35.0	1,855	34.7	2,578	32.8	

*Comparison of all 3 rosters.

moved very differently over time. For all 3 rosters, deaths from pneumonia were at their peak in 1929–38 and fell off rapidly thereafter, while deaths from other respiratory diseases increased over this period. The high death rates for respiratory diseases in 1929–38 are thus entirely attributable to pneumonia.

General Review of Mortality

Other causes of death were reviewed by means of a two-digit classification of primary cause of death. Table 6 provides data which compare rosters for mortality from broad groups of causes. In addition to those groups of causes that have already been discussed, significant variation is noted for only two: tuberculosis of the respiratory system ($P<0.01$) and diseases of the nervous system or sense organs ($P = 0.03$).

Table 7 shows tuberculosis death rates for the 3 rosters as functions of calendar time and provides further information on the cause of the abnormal rise in the death rate of men on the mustard-gas roster during the decade 1929–38: Not only pneumonia but also tuberculosis contributed to that increase. The table also shows why the death rate for men on the pneumonia roster was abnormally high at the very outset of the follow-up period: In the decade 1919–28 the death rate for tuberculosis among men on this roster was 1.28 (23 deaths) and suggests that at least 10 of the 1,855 men on this roster actually had acute tuberculosis (of which disease they subsequently died) at the time they were thought to have had pneumonia. To compare tuberculosis mortality in these samples with that which would have been expected from rates for U.S. white males, suitable rates were developed from a summary (*23*) published by the National Office of Vital Statistics.

TABLE 7.—Number of deaths and deaths per 1,000 men per year for respiratory tuberculosis, by roster, and by period of exposure

Period of exposure	Mustard-gas		Pneumonia		Wounded controls	
	Number of deaths	Rate	Number of deaths	Rate	Number of deaths	Rate
1919–28	17	0. 64	23	1. 28	15	0. 59
1929–38	41	1. 63	12	0. 70	13	0. 54
1939–48	27	1. 20	15	0. 96	19	0. 86
1949–55	14	1. 03	4	0. 43	7	0. 53
Total	99	1. 13	54	0. 89	54	0. 63

Table 8 presents the resulting data by age. During the first decade, although the observed mortality was about half of expectation for the mustard-gas and control rosters, it was slightly above expectation for the pneumonia roster. One might conclude that military screening depressed the death rate from tuberculosis for this period, but that errors in diagnosis (tuberculosis mistaken for pneumonia) account for the deviant behavior of the pneumonia roster. The data suggest that, in the control roster, the effect of medical selection progressively diminished to zero during the second and third decades. For the pneumonia roster, on the

other hand, by the second decade after selection the tuberculosis death rate had returned to general population levels, there to remain. The suggestion is strong that the tendency occasionally to misdiagnose tuberculosis as pneumonia resulted in selection for this disease in men on the pneumonia roster. On this view, the agreement between observed and expected mortality is merely a coincidence: The influence of this error in selection offsets the effect of medical screening.

TABLE 8.—Observed deaths from tuberculosis, all forms, and expected deaths based on experience of U.S. white male population of comparable age at calendar time of exposure

Age	Mustard-gas		Pneumonia		Wounded controls	
	Observed	Expected	Observed	Expected	Observed	Expected
26–34	11	23.7	19	16.0	12	22.5
35–44	39	23.9	16	16.2	15	22.8
45–54	27	19.6	13	13.6	15	19.2
55–	25	13.1	10	9.1	14	12.9
Total	102	80.3	58	54.9	56	77.4

The experience of men on the mustard-gas roster is most atypical, and one cannot escape the conclusion that some interaction occurred between the mustard-gas injury, or its residuals, and tuberculosis. Although the experience of the first decade seems normal enough, thereafter men on the mustard-gas roster consistently died from tuberculosis more often than would be expected in relation to the civilian population; the excess amounts to 34 deaths above the expected 57. The excess is even greater with respect to the control roster. It may be remarked incidentally that none of the 5 cases of lung cancer found at autopsy, but not so diagnosed on the death certificate, occurred in an individual whose death certificate stated tuberculosis as the cause.

The only other causes to show any evidence of heterogeneity in table 6 are diseases of the nervous system and sense organs. Primarily, this rubric represented cerebral vascular disease; just over half of all deaths were attributed to "cerebral hemorrhage." The variation among rosters is not remarkably large: the calculated probability of a sampling result as extreme as that observed is about 1 in 30, on the hypothesis of homogeneity. Inspection of data on individual causes revealed no marked discrepancy for any single one; it is only when all are added that the low count for the mustard-gas roster attracts attention. The variation is localized in time to the last decade; at a time when rates for men on the pneumonia and control rosters were doubling, the rate for men on the mustard-gas failed to rise sharply. Since, directly and by implication, hundreds of such comparisons have come under scrutiny here, it may well be that this particular discrepancy is merely a chance event.

In addition to the individual causes that have already been discussed because of either intrinsic interest or their dominance of a large class of deaths in which roster variation was observed, those individual causes were examined for roster variation which accounted for at least 16 ob-

served deaths in the 3 rosters combined. There were 15 such causes, but for only one—cancer of the pancreas—was there apparently significant roster variation. Of the 16 deaths observed, 12 were in the mustard-gas roster, and the remaining 4 in the controls. This distribution of the 16 deaths by roster is a most unlikely one (P about 0.005).

BRONCHITIS AND LUNG CANCER

Information on bronchitis has been sought in this study because Case and Lea indicated that any excess of lung cancer attributable to mustard gas in their British series probably stems from the fact that the men in their mustard-gas sample all drew pensions, chiefly for chronic bronchitis. Earlier studies (*24*) on U.S. veterans with mustard-gas injury in World War I have indicated that chronic bronchitis is a common sequel of such injury.

In the expectation that a relationship between bronchitis and lung cancer would be found in this series, and that its maximum strength would be exhibited in mortality ratios based on deaths forward of a given calendar date before which bronchitis had already manifested itself, tabulations were centered on various calendar dates. For each tabulation date, separately for men who subsequently died of lung cancer and for those who did not, these tables show which men already had bronchitis, which men first had bronchitis later, and which men never had bronchitis. Table 9 provides a summary of these data for the 1926, 1936, and 1946 tabulation points, and shows that evidence of a relationship between bronchitis and lung cancer is completely lacking. There are important roster differences in the reported prevalence of bronchitis, but they pertain equally to lung-cancer deaths and to others. For the mustard-gas and control rosters, with their more stable numbers of cases, the percentages involved in the horizontal comparisons are especially close.

Why the U.S. material should differ from the British has no answer in these data, but one would at first suspect that either chronic bronchitis has very different significance in Great Britain or, possibly, that sampling bias has crept into one or the other of the studies.

Bronchitis and Roster Conditions

As indicated, the rosters vary quite significantly as to reported prevalence of bronchitis. However, since each roster was chosen on the basis of a specific item of medical history during military service, and in this study the morbidity information was developed from VA claims folders, it is to be feared that the direct comparison of the rosters may be biased. The objectives of the study did not include comparison of men on the rosters as to morbidity, and, accordingly, the only measures of relative bias are quite indirect. With respect to bronchitis, it may be mentioned that morbidity, as measured by reported admissions to service or VA

TABLE 9.—Relation between lung cancer and bronchitis, by roster, for various calendar periods of observation

Calendar period			Deaths from lung cancer			Other deaths and survivors to 1956 in 10-percent sample			
				Bronchitis present			Bronchitis present		
For deaths	For first report of bronchitis	Roster	Total	Number	Percent	Total	Number	Percent	*P*
1926–55	1919–55	Mustard-gas	36	21	58. 3	264	157	59. 1	>0. 05
		Pneumonia	13	6	46. 2	174	58	33. 9	>0. 05
		Controls	26	5	19. 2	247	48	19. 4	>0. 05
1926–55	1919–25	Mustard-gas	36	14	38. 9	264	105	39. 8	>0. 05
		Pneumonia	13	3	23. 1	174	27	15. 5	>0. 05
		Controls	26	2	7. 7	247	21	8. 5	>0. 05
1936–55	1919–35	Mustard-gas	35	18	51. 4	242	124	51. 2	>0. 05
		Pneumonia	13	5	38. 5	163	38	23. 3	>0. 05
		Controls	26	3	11. 5	235	37	15. 7	>0. 05
1946–55	1919–45	Mustard-gas	29	15	51. 7	210	119	56. 7	>0. 05
		Pneumonia	9	5	55. 6	146	38	26. 0	>0. 05
		Controls	26	5	19. 2	208	35	16. 8	>0. 05

hospitals with this diagnosis, bore essentially the same ratio to mortality from this cause (primary or associated) in the 3 rosters. With this *caveat*, table 10 shows the continuing build-up of cases by roster, starting in 1919–20, when one fourth of all men on the mustard-gas roster registered evidence of bronchitis, through 1957.

TABLE 10.—Cumulative percentage distributions of men as to first diagnosis of bronchitis, 10-percent sample, by roster

Period of first diagnosis	Roster		
	Mustard-gas	Pneumonia	Controls
	(*Cumulative percentage*)		
1919–20	24.5	8.1	3.0
1921–25	43.1	16.8	9.4
1926–30	49.4	18.6	12.4
1931–35	56.5	24.8	16.7
1936–40	59.7	28.0	17.6
1941–45	61.7	28.6	17.6
1946–50	62.8	31.7	18.9
1951–55	63.2	34.2	20.6
1956–57	63.6	36.0	21.0
Number of men (folders reviewed)	253	161	233

Men on the mustard-gas roster not only reported more bronchitis—their onset was much earlier.

CONCLUSIONS

Two problems in the epidemiology of lung cancer have been attacked on the basis of a mortality study of U.S. Army veterans of World War I: the 1918 influenza epidemic and war injuries attributed to mustard gas. The basic design is that of a prospective study initiated entirely on the basis of military records created in 1918, aimed at the detection of 3 : 1 differences between samples of approximately equal size compared as to mortality from lung cancer. Representative rosters of 2,718 white men with mustard-gas injury, 1,855 with pneumonia in 1918, and 2,578 with wounds of the extremities (controls), were created from retired Army records in the St. Louis record depot. Year of birth was confined to 1888–93, inclusive. The original Army clinical records of mustard-gas injury were reviewed according to criteria thought sufficient to insure the validity of the diagnosis and the determination of the responsible agent. The entire roster of 7,151 men was then traced forward through 1955 on the basis of records of insurance, hospitalization, and disability maintained by the Veterans Administration.

Soon after the study was begun, a report became available by Case and Lea on 1929 British Army pensioners with residuals of mustard-gas injuries received in World War I. Their report suggested that men with mustard-gas injury had about twice the expected incidence of lung cancer, but only because of an intervening residual in the form of chronic bron-

chitis. In view of this report the design of the present study was modified to obtain data on VA hospitalizations, outpatient visits, and disability ratings for 10 percent of the sample.

The total of 2,441 deaths reported through 1955 was 95 percent of expectation on the basis of age- and time-specific death rates for U.S. white males over this interval. While in the first decade only 71 percent of the expected deaths were found, in the period 1940–55 the proportion of expected deaths was 98 percent. It is believed that the early deficit represents the influence of screening for military service in World War I, and that VA reporting of deaths is substantially complete.

The 3 rosters differ significantly as to gross mortality only in the second decade of the follow-up period, when men on the mustard-gas roster suffered from considerably higher mortality rates. Examination of the deaths by cause revealed that this excess arose primarily from pneumonia and tuberculosis.

The data on mortality from lung cancer are equivocal with respect to the influence of mustard-gas injury, but contain no hint that postinfluenzal pneumonia in the 1918 fall epidemic increased the risk of lung cancer. Observed lung-cancer deaths are 36/2,718, or 1.32 percent, for the mustard-gas roster, and 26/2,578, or 1.01 percent, for the control roster. These percentages do not differ significantly and thus provide but weak support for the view that mustard-gas injury sets the stage for the later development of bronchogenic cancer. However, the study was planned to have good power against a 3 : 1 ratio but not against a 2 : 1 ratio, and since Case and Lea reported only a 2 : 1 ratio in their material, the present finding is not inconsistent with their conclusion. It seemed essential, therefore, to make a supplementary comparison of observed mortality in relation to expectation based on the general U.S. population experience for white males. This can be done for all respiratory cancer and provides a more powerful, if less direct, comparison. In these terms the ratios of observed to expected deaths are 39/26.6, or 1.47, for the mustard-gas roster, 15/18.6, or 0.81, for the pneumonia roster, and 30/26.2, or 1.15, for the control roster. Only the first differs significantly ($P < 0.05$) from unity. Although the study was not designed on the basis of this approach, because a direct comparison of two sociologically similar samples, followed up in the same way, seemed distinctly preferable, correspondence between veteran and civilian mortality seems sufficiently close to invest this indirect comparison with more merit than could be assumed for it in the planning stage. As originally planned, therefore, the study renders a verdict of "unproved," but the indirect comparison with population values is quite suggestive that an association exists. However, it is not consistent with the view that the increment of risk is large, relative to that existing in the entire control population, the 95 percent confidence interval on the observed ratio of 1.47 being 1.02 to 1.96. The Case and Lea result of 2.07 falls just outside this confidence interval. To the extent that cigarette smoking could be an important source of risk for the control population, for nonsmokers the relative increment of risk associated with a single mustard-gas exposure could, nevertheless, be large.

The relationship between bronchitis and lung cancer was studied directly by comparing the percentages exhibiting bronchitis at a fixed date, *e.g.*, January 1, 1926, in (*a*) men who later died of lung cancer, and (*b*) men who did not later die of cancer. This was done for each roster and for a quinquennial series of dates, but no evidence of association was found. Large roster differences were observed, however, in the reported incidence of bronchitis, in that about 65 percent of the VA records of men on the mustard-gas roster, 35 percent on the pneumonia roster, and 20 percent on the wounded roster, showed evidence of bronchitis. The failure of these data to confirm those of Case and Lea, therefore, suggests that bronchitis may not be a uniform clinical entity in Britain and the United States.

Deaths from neoplasms of the lymphatic and hematopoietic systems were few and did not vary among the several rosters. There were 14 deaths from leukemia and aleukemia, almost evenly divided among the 3 rosters. The experience with respect to other cancers was not remarkable, except for cancer of the pancreas: 12 of the recorded 16 deaths were among men on the mustard-gas roster and none on the pneumonia roster ($P<0.01$). Deaths from the anemias were also infrequent and did not vary by roster.

Deaths from diseases of the nervous system and sense organs also differ significantly ($P = 0.03$) among the 3 rosters, the discrepancy favoring the mustard-gas sample. In view of the large number of significance tests performed here, a discrepancy of this degree of improbability (1/30) may well be an accident of sampling.

Deaths from tuberculosis were much more frequent among men on the mustard-gas roster than among men on the wounded controls roster (3.8 vs. 2.2%), with the excess being experienced only after age 35. Before age 35 the tuberculosis mortality experience was about the same in both groups and about half that of U.S. white males generally.

Pneumonia, like tuberculosis, caused significantly greater mortality in the mustard-gas roster than in controls (2.9 vs. 1.4%). However, in this instance the difference varied little in time.

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Neoplasms Among A-Bomb Survivors in Hiroshima: First Report of the Research Committee on Tumor Statistics, Hiroshima City Medical Association, Hiroshima, Japan [1, 2, 3]

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SUMMARY

The 1957–58 incidence of neoplasms among the survivors of the Hiroshima A-bomb varies directly with radiation dose, insofar as it may be inferred from distance from the hypocenter at exposure. The incidence of all malignant neoplasms among the survivors who were within 1000 meters is more than 4 times that of the nonexposed population. The incidence of benign neoplasms among the survivors exposed within 1500 meters is also significantly higher than that among the nonexposed. For survivors under 1500 meters there are significant differences between the number of observed cancers of the lung, stomach, uterus, and ovary and the expected cases calculated from the age-specific rates of the nonexposed portion of the Hiroshima population. The increased incidence among survivors within 1500 meters is not related to sex or age.—J. Nat. Cancer Inst. 25: 1253–1264, 1960.

THIS PAPER presents an analysis of the incidence of malignant neoplasms diagnosed during May, 1957, to December, 1958, in survivors of the 1945 atomic bombing of Hiroshima. The series of papers on leukemias among A-bomb survivors in Hiroshima (*1–4*) and the British report by Court-Brown and Doll (*5*) on leukemias among ankylosing spondylitis

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treated by large doses of X ray have definitely established a positive correlation between leukemia incidence and irradiation. Experiments with animals have shown that ionizing radiation can produce neoplasms in almost any tissue (*6–10*). The Hiroshima survivors are unusual in having experienced a single whole-body exposure, the actual dose varying according to distance from hypocenter and shielding condition. Two previous reports on the incidence of cancer in this population have appeared: Murphy and Yasuda (*11*) found no difference in the incidence of cancer of the stomach between those exposed within 2500 meters from the hypocenter and those not exposed; and Oho (*12*), in a death-certificate analysis, demonstrated an approximately 40 percent higher mortality among the survivors exposed within Hiroshima City in comparison with the nonirradiated. The present investigation is designed to correlate the incidence of neoplasms among the Hiroshima A-bomb survivors with the amount of radiation received. The data used for this analysis were obtained during the first 20 months of operation of the Tumor Registry maintained by the Hiroshima City Medical Association. The many cases received at the tumor registry at the time of its commencement were carefully reviewed, and approximately 100 registered cases were discarded from the study because diagnosis was made before May 1, 1957, the official starting date.

HIROSHIMA TUMOR REGISTRY

To study the relation between malignant neoplasms and radiation doses, the Hiroshima City Medical Association established a community Tumor Registry with the financial and technical assistance of the Atomic Bomb Casualty Commission (ABCC) and the Japanese National Institute of Health (NIH), in accordance with a suggestion of Dr. Robert H. Holmes, former Director of ABCC. The Registry, which serves all of Hiroshima City, began functioning on May 1, 1957, in compliance with a resolution passed by the General Assembly of the Hiroshima City Medical Association on January 24, 1957. A research committee on tumor statistics was created within the Association to manage the Registry. All tumor cases, both malignant and benign, diagnosed in hospitals or clinics within Hiroshima City limits were reported to the Association. The history of exposure was obtained from ABCC records which reflect extensive field investigation of survivors over many years. The data, therefore, provide basic information on the exposed and the unexposed segments of the population of Hiroshima. Approximately 1,750 cases of malignant neoplasms and 810 cases of benign tumors were registered in the 20-month period.

ACCURACY OF DIAGNOSIS

All registered cases were unduplicated and checked against the surgical, pathology, and autopsy records of the Hiroshima Hospital of the Japan

Red Cross Society, the Hiroshima Prefectural Hospital, the Hiroshima Medical School, and ABCC. As shown in table 1, approximately 58 percent of the malignant neoplasms in both sexes were confirmed by microscopic examination of tissues taken at biopsy or autopsy. The cases diagnosed by clinical examination alone and cases based only on death certificates have been considered "unconfirmed"; they approximate 30 percent of the registered cases. The remainder, diagnosed by X ray or surgical operation, has been considered "confirmed."

TABLE 1.—Percentage distribution of registered cases by method of diagnosis and by sex, May, 1957 to December, 1958

Type and site of neoplasms	Number*	Method of diagnosis					
		Clinical	X ray	Operation	Biopsy	Death certificate	Autopsy
Males							
Malignant neoplasms.....	409	4.6	11.7	3.2	41.8	26.2	12.5
Benign neoplasms...	82	28.0	11.0	2.5	57.3	—	1.2
Cancer of stomach..	203	3.4	16.8	4.4	34.5	30.1	10.8
Females							
Malignant neoplasms.....	531	6.6	8.1	3.6	53.1	19.9	8.7
Benign neoplasms..	294	18.4	2.7	28.2	49.7	0.3	0.7
Cancer of stomach..	140	3.6	22.9	2.8	33.6	30.0	7.1
Cancer of uterus....	111	1.8	—	1.8	82.9	9.9	3.6
Cancer of breast....	47	2.1	—	2.1	87.3	6.4	2.1
Cancer of ovary....	20	15.4	—	15.4	53.8	7.7	7.7

Table 2 gives percentages according to the method of diagnosis among the exposed patients classified by distance from the hypocenter. No remarkable differences were observed.

TABLE 2.—Percentage distribution of registered malignant neoplasms according to distance from hypocenter by method of diagnosis, May, 1957 to December, 1958*

Distance from hypocenter (*meters*)	Number	Method of diagnosis					
		Clinical	X ray	Operation	Biopsy	Death certificate	Autopsy
500–999	19	5.3	5.3	—	36.8	15.8	36.8
1000–1499	77	2.6	6.5	3.9	45.5	26.0	15.6
1500–1999	100	6.0	11.0	4.0	44.0	24.0	11.0
2000–2499	78	5.1	10.3	2.6	41.0	20.5	20.5

*Includes cases that occurred among people living outside the city.

To evaluate the unsupported clinical diagnoses, we correlated autopsy diagnoses made at ABCC from 1948–59 with the previous clinical diagnoses of cancer—94 percent were confirmed at autopsy; of 325 autopsy diagnoses of cancer, 76 percent had been so diagnosed. This operation indicates that the correlation is good and that clinical diagnoses of malignancies are reasonably reliable. Therefore, the present analysis makes use of both confirmed and unconfirmed cases in the Registry.

INCIDENCE OF MALIGNANT NEOPLASMS IN HIROSHIMA CITY

The incidence of cancer obtained from the Registry was adjusted to the 1955 census of population of Hiroshima, both sexes combined. For comparison, the rates in Miyagi Prefecture, Japan (*13*), in Connecticut, U.S.A. (*14*), and in Copenhagen, Denmark (*15*), were standardized to the same population. These results are shown in table 3.

TABLE 3.—Annual numbers of malignant neoplasms, all types, per 100,000 population by sex in various areas in the world; rates standardized against 1955 Hiroshima population

	Male	Female
Hiroshima City, 1957–58	150. 2	168. 9
Miyagi Prefecture, 1951–52	125. 3	116. 4
Connecticut, 1947–51	159. 0	166. 5
Copenhagen, 1943–47	144. 1	160. 9

In Hiroshima City the incidence of malignant neoplasms was almost the same as in Connecticut and Copenhagen and considerably higher than in Miyagi Prefecture, which supports the view that the Registry is a valid tool for studying tumor incidence in Hiroshima.

CANCER INCIDENCE IN A-BOMB SURVIVORS

General

Air-dose curves and attenuation factors for various shielding materials have been estimated by physicists from experimental data and from information on the bomb composition and its height at the time of explosion (*16*). Calculation of accurate radiation doses for individual survivors requires, in addition, detailed shielding histories which at this time are not sufficiently complete to permit calculation on a scale sufficient to warrant their use in this study. However, it is worthy of mention that 90 percent of the survivors exposed within 2000 meters from the hypocenter were in the open or in Japanese wooden houses when the bomb exploded.[5]

In view of the difficulties in estimating the current population of the survivors, as discussed in the latter part of this report, rates were calculated not only for the estimated population of Hiroshima City in 1957,[6] but also for the fixed sample used in the NIH-ABCC mortality study (Master Sample).[7]

[5] Individual shielding histories of persons exposed near the hypocenter are being systematically collected by ABCC and will permit the use of radiation dose in future analyses.

[6] *Estimated current population:* The survivor population of 1955 was estimated from the 1950 exposed population by age and sex, listed on the supplementary schedules of the Japanese National Census, by applying the survival rate of the 9th Official Life Table and the hypocenter distance distribution obtained from the ABCC Radiation Census, 1949.

[7] *Master Sample:* The Master Sample is a fixed sample used by the Japanese NIH and ABCC in their joint study of mortality in the A-bomb survivors. Information on malignant neoplasms is, of course, limited to those who were diagnosed by physicians in Hiroshima City. Having as its basis residence in Hiroshima in 1950, the Master Sample is concentrated in this city, but migrants have scattered throughout the country. Therefore, use of the Master Sample leads to an underestimation by the number of unreported cancer cases in persons who are no longer in the Registry area.

The sample exposed beyond 2500 meters from the hypocenter has been omitted from this analysis because of difficulties in estimating the number of the sample and in obtaining the cancer information developed from the sample. A considerable portion of the sample was occupied by the survivors who lived outside the city limit at the time of bombing.

Incidence of All Neoplasms

Tables 4 and 5 give two different estimates of the incidence of all malignant neoplasms by distance from the hypocenter. Both methods clearly demonstrate a relationship between distance from the hypocenter and incidence. The difference between the rates for persons exposed at short distances from the hypocenter, *i.e.*, within 1500 meters, and for the unexposed is highly significant. The rate within 1000 meters from the hypocenter is four times that of the unexposed. The incidence based on the Master Sample is approximately 30 percent lower than that based

TABLE 4.—Number of all malignant neoplasms and samples by age, sex, and distance in meters from hypocenter based on current estimated population, May 1, 1957–December 30, 1958 (crude and adjusted rate per 100,000 by distance from hypocenter are illustrated)

Age	Distance from hypocenter*					Population of Hiroshima City‡
	500–999	1000–1499	1500–1999	2000–2499	Unexposed†	
Male						
10–19	0/50	1/683	0/1509	0/1543	(2)4/27,823	40,724
20–29	0/81	1/793	1/1579	1/790	(3)8/25,913	36,401
30–39	0/41	0/346	(1)1/573	0/448	(2)12/22,075	26,893
40–49	(1)1/70	1/523	1/797	3/735	50/15,945	22,417
50–59	(1)2/83	6/719	11/965	5/974	56/7381	14,103
60–69	3/52	(1)14/427	(1)18/684	(1)15/708	40/2837	7025
70–79	(1)3/11	7/140	12/266	7/286	27/1089	2924
80–over	0/1	0/18	0/45	0/40	5/235	567
Total	(3)9/389	(1)30/3649	(2)44/6418	31/5524	202/103,298	151,054
Female						
10–19	0/47	0/708	1/1465	0/1580	(1)7/25,913	38,680
20–29	1/208	(1)1/1060	1/1891	0/1593	(2)14/27,499	39,359
30–39	1/151	(1)4/978	5/1548	(1)3/1357	(2)45/20,933	31,821
40–49	2/79	(1)5/876	3/1511	4/1448	(5)65/12,583	22,311
50–59	2/56	13/789	16/1348	10/1236	(3)57/5974	13,862
60–69	1/27	(1)12/404	11/700	10/717	36/3735	7938
70–79	0/12	5/180	10/350	9/375	28/2155	4597
80–over	0/4	1/35	0/87	2/85	10/637	1395
Total	7/584	(4)41/5039	47/8900	38/8391	262/99,429	159,963
Grand total	(3)16/973	(5)71/8688	(2)91/15,318	(2)69/13,915	(20)464/202,727	311,017
All malignant neoplasms						
Crude rate per 100,000	1644	817	594	496	229	
Age and sex-adjusted rate	1287	529	393	297	280	
Malignant neoplasms excluding leukemias and lymphomas						
Crude rate per 100,000	1336	760	581	474	219	
Age and sex-adjusted rate	1023	479	380	270	271	

*Numbers in parentheses show cases of leukemias and lymphomas; 0/50 = no malignant tumor cases among 50 sample members.

†"Unexposed" is defined as those who were more than 10,000 meters from the hypocenter at the time of bombing.

‡1955 Census Population of Hiroshima City, which was used as the standard population for calculation of adjusted incidence rates.

TABLE 5.—Number of all malignant neoplasms and samples by age and distance in meters from hypocenter based on the Master Sample, sexes combined May 1, 1957–December 30, 1958 (crude and adjusted rates per 100,000 are lillustrated)

Age	Distance from hypocenter			
	500–999	1000–1499	1500–1999	2000–2499
10–19	0/110*	1/1310	1/2227	0/2552
20–29	0/221	1/1374	2/2558	0/1957
30–39	1/178	2/1032	2/1559	1/1341
40–49	3/134	4/1256	2/1834	10/1659
50–59	5/144	17/1428	20/1974	9/1768
60–69	1/85	13/902	19/1342	21/1277
70–79	1/34	6/418	13/703	8/697
80–over	0/6	1/98	0/188	1/214
Total	11/912	45/7818	59/12,385	50/11,465
Crude rate per 100,000	1,206	576	476	436
Age and sex-adjusted rate	864	336	274	256

*Indicates no malignant tumor cases among 110 sample members.

on the current population, but the same inverse correlation between incidence and distance from the hypocenter is manifest. Cases of leukemia and lymphoma were excluded and incidence recalculated. The results remain quite definite.

Incidence by Sex

The incidence of all malignant neoplasms among females exposed within 1500 meters is higher than that for males. However, tumor incidence is also higher among females in the unexposed portion of the Hiroshima population. The ratios of the exposed to the unexposed rates are 1.9 for males and 2.0 for females, where the exposed are confined to those under 1500 meters from the hypocenter at the bombing (table 6).

TABLE 6.—Adjusted incidence of the total malignant neoplasms by sex, May 1, 1957 to December 31, 1958, based on the current population

Exposure	Male	Female	Sex ratio
Irradiated within 1500 meters from hypocenter	533. 1	610. 0	1. 14
Nonirradiated	274. 3	306. 3	1. 12

Incidence of Malignant Neoplasms by Site

A detailed analysis of the site-distribution of malignant neoplasms is not practicable on the basis of the limited number of cases obtained during the first 20 months since the initiation of the Registry. Table 7 shows the number of observed and expected cases of cancer of selected sites among persons within 1500 meters from the hypocenter. We calculated the expected numbers by applying to the estimated sample of the exposed the age and sex-specific incidence of neoplasms observed for the unexposed population in Hiroshima. Chi-square tests on major sites of cancer by the method of Mantel and Haenszel (*17*) show significant

differences between cases classified by distance from hypocenter and the unexposed.

TABLE 7.—Comparison of observed and expected cases of malignant neoplasms of selected sites among individuals in the current city population exposed within 1500 meters from the hypocenter, April, 1957 to December, 1958*

	Observed	Expected	Ratio	Test result
Cancer of stomach, sexes combined	24	12. 41	1. 93	†
Cancer of lung, sexes combined	10	2. 32	4. 31	†
Cancer of breast	5	2. 49	2. 00	‡
Cancer of cervix uteri	8	4. 99	1. 60	§
Cancer of ovary	4	1. 01	3. 96	‡

*Results of the chi-square test mentioned by Mantel and Haenszel (17) show significant findings in each site of cancer except cancer of the cervix uteri.
†Significant at the level of 1 percent.
‡Significant at the level of 5 percent.
§Not significant.

Age-Specific Incidence of Cancer in A-Bomb Survivors

The log of incidence increases as a linear function of age in both the exposed within 1500 meters and the unexposed, as shown in text-figure 1. Excluding leukemias and lymphomas, which are prevalent in the younger age group and are definitely increased by radiation, the ratio of the exposed to the unexposed incidence is approximately the same for all age groups. No specific age group appears particularly susceptible to the induction of neoplasms by irradiation (text-fig. 1).

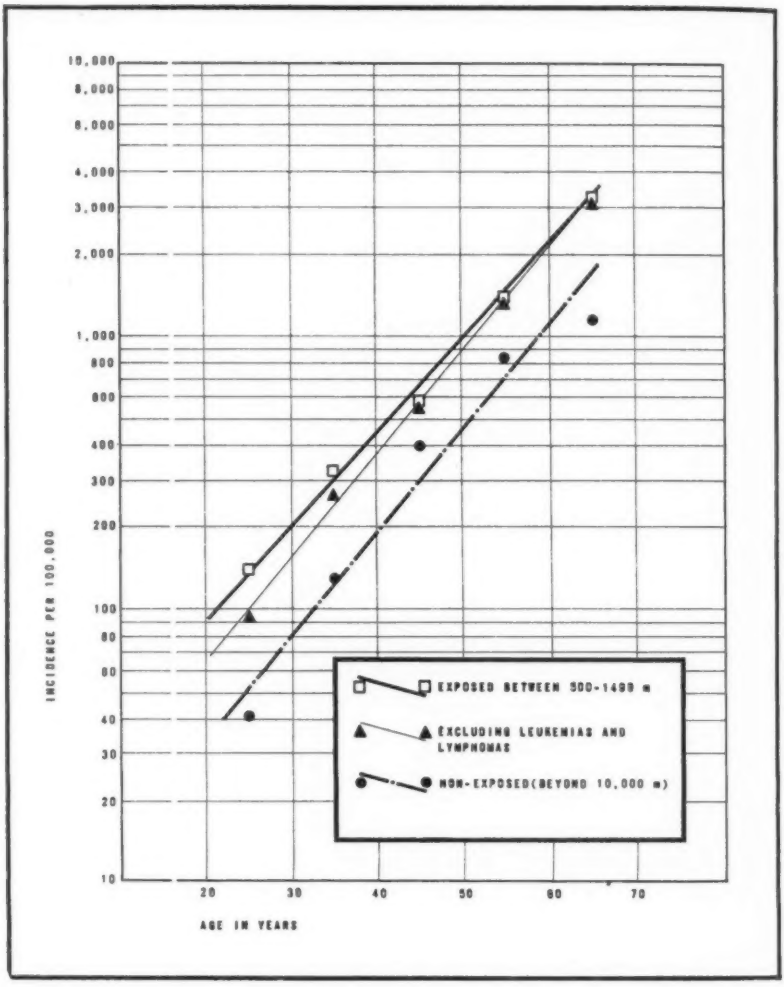
Incidence of Benign Neoplasms

Table 8 shows the incidence of benign neoplasms by distance from the hypocenter. A significant difference is observed between the rates for

TABLE 8.—Number of all benign neoplasms and samples by age and distance in meters from hypocenter based on the current estimated population, sexes combined May, 1957 to December, 1958 (crude and adjusted rates per 100,000 are illustrated)

	Distance from hypocenter				
Age	500–999	1000–1499	1500–1999	2000–2499	Unexposed
10–19	0/97*	1/1391	0/2974	0/3123	38/53, 736
20–29	0/289	2/1862	4/3470	1/2383	24/53, 412
30–39	0/192	6/1324	6/2121	4/1805	58/43, 008
40–49	1/149	6/1399	5/2308	4/2183	67/28, 528
50–59	0/139	0/1508	5/2313	6/2210	49/13, 355
60–69	1/79	3/831	1/1384	5/1425	33/6572
70–79	1/23	0/320	0/616	1/661	8/3244
80–over	0/5	0/53	0/132	1/125	2/872
Total	3/973	18/8688	21/15, 318	22/13, 915	279/202, 727
Crude rates per 100, 000	308	207	137	158	138
Age and sex-adjusted rates	225	210	136	159	122

*Indicates no benign tumor cases among 97 sample members.



TEXT-FIGURE 1.—Incidence of malignant neoplasms by age and exposure status. May 1, 1957 to December 30, 1958, Tumor Registry, Hiroshima.

the exposed within 1500 meters and the unexposed. However, the number of benign neoplasms available from the present study is far too small for analysis by site.

DISCUSSION

This analysis has demonstrated a higher incidence of malignant neoplasms among A-bomb survivors exposed near the hypocenter. Murphy and Yasuda (*11*), of the Atomic Bomb Casualty Commission in Hiroshima,

reported that no increase in incidence in stomach cancer among A-bomb survivors could be demonstrated. It is suggested that these researchers may have failed to detect a significant difference in incidence because of insufficient sample size and an inefficient grouping of distance from the hypocenter.

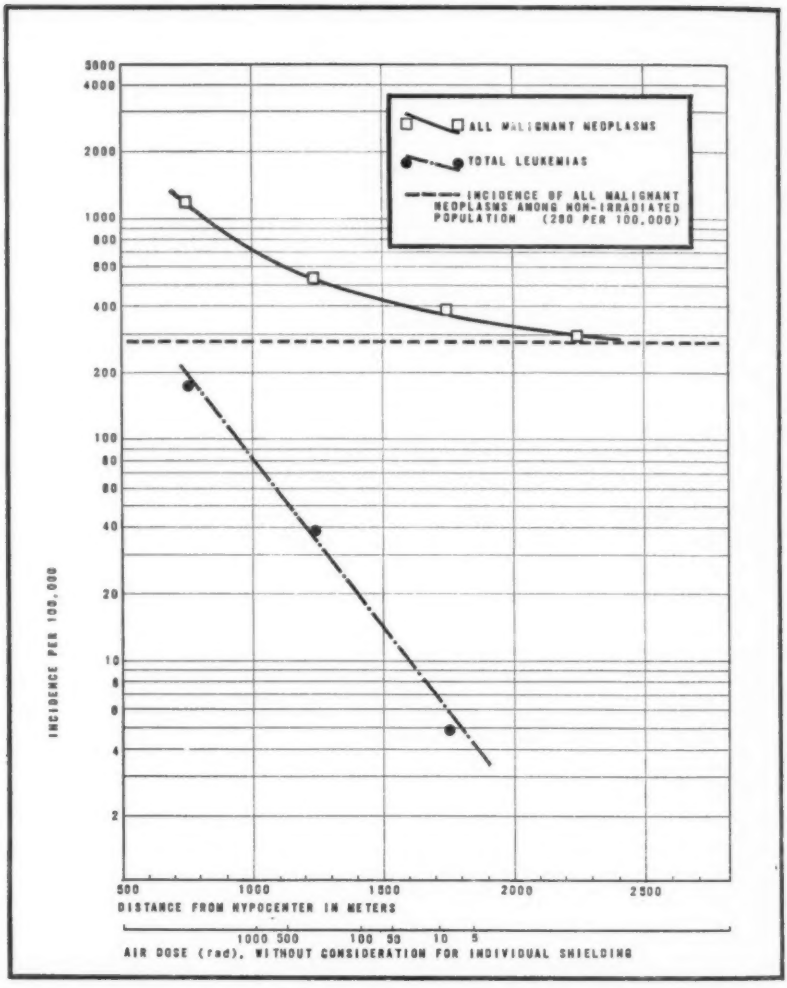
This analysis of Tumor-Registry cases is based on two kinds of population estimates: (*a*) the current population estimated from the 1950 National Census by the use of the life-table survival rate in Japan and ignoring migration, and (*b*) a fixed sample selected for the mortality study conducted by ABCC and Japanese National Institute of Health. Since animal experiments have demonstrated a lifespan shortening in parallel radiation doses (*18*), overestimation of the surviving population under 1500 meters may result from the use of the life-table mortality rate (*9*) with consequent underestimation of the incidence of neoplasms in survivors close to the hypocenter. The general validity of the analysis based on the current population seems confirmed by the results in a fixed sample.

Since the initial mortality in 1945 was so high, the survivors located within 1000 meters of the hypocenter at the time of bombing must have received doses ranging up to the maximum tolerable dose for man. The incidence of all malignant neoplasms in the sample, some of whom were exposed to the *maximum sublethal dose by a single external whole-body irradiation*, can be estimated from the data collected by the Registry. The annual incidence was found to be approximately 1,200 per 100,000, which is more than 4 times that of the unexposed population. The so-called "doubling dose" for human cancer is observed at approximately 1300 meters from the hypocenter. The equivalent *air dose* is approximately 400 rads (*16*), equal to or somewhat below the LD50 for human beings (*19*).

Whether the carcinogenic effect of radiation is a linear function of dose is somewhat illuminated by this analysis. The most recent ABCC analysis of leukemia suggests that the incidence of leukemia among A-bomb survivors in Hiroshima increases as a linear function of apparent radiation dose. The impression obtained from the present analysis is that the incidence of all malignant neoplasms increases as a function of dose, exponential or linear, as shown in text-figure 2. The abscissa and ordinate in text-figure 2 show incidence on a logarithmic scale and the distance from the hypocenter on an arithmetic scale; however, the latter is equivalent to radiation dose on a logarithmic scale. If incidence increases as a linear function of dose, the distance-specific incidence in text-figure 2 also should be a straight line. Further analysis on this point is necessary to establish a definite conclusion, for the series is still small and accurate shielding information on each survivor is still lacking.

The data of the Registry indicate that the incidence of benign neoplasms among survivors exposed within 1500 meters of the hypocenter was 2.0 times that of the unexposed during May, 1957, through December, 1958.

Two alternative hypotheses concerning the nature of the radiation effect have been advanced in the literature. A true carcinogenic effect



TEXT-FIGURE 2.—Incidence of all neoplasms among A-bomb survivors, May 1, 1957 to December 30, 1958, Tumor Registry, Hiroshima, and incidence of total leukemia among residents in Hiroshima City at onset, January, 1950 to December, 1957 (*4*).

has been postulated, but an alternative explanation is that radiation simply accelerates the appearance of neoplasms without increasing their lifetime incidence. The data of the Registry seem to support the hypothesis that the increase is absolute, not a mere shift in age-specific incidence. This follows from the observation that the age-specific rates of malignant neoplasms, excluding leukemias and lymphomas, for survivors exposed within 1500 meters, are a constant multiple of the corresponding rates for the unexposed and do not reveal a mere shift to the younger ages. How-

ever, this observation is based on the present cross-section study, and to establish a definitive conclusion a longer period of study seems necessary.

The apparent effect of radiation, as seen in those exposed under 1500 meters, differs by site and by type of malignancy. This evidence suggests two alternative hypotheses as to the occurrence of carcinogenic action by radiation: (*a*) Specific organs or tissues differ in their radiosensitivity, and (*b*) specific sites differ as to time lag from exposure to the development of tumor. These alternatives cannot be investigated with the short-term data now available. The changes with time in the incidence of tumors generally, and of the various organs individually, must be elucidated through continued data collection by the Registry.

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Study of Methods for Abbreviating Carcinogenicity Bioassays. I. Enhancement of Neoplastic Response by Pretreating With a Potent Carcinogen [1, 2, 3]

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SUMMARY

A study was made of the biologic summation of carcinogenic effects induced by serial application of active agents to the skin of mice. Limited pretreatment of the interscapular skin with measured amounts of benzo[*a*]pyrene in toluene, followed by chronic applications of less potent "test" carcinogenic solutions, reduced the time preceding tumor appearance and augmented the incidence of tumor-bearing animals obtained with either procedure alone. The observed pattern of tumor induction supports the concept that a tumor is a biologic response to the sum of effective carcinogenic stimuli received by a tissue throughout its preneoplastic state. The data suggest the feasibility of summating the effects of calibrated exposures to a potent carcinogen with that of subsequently applied materials of unknown potency as a technique for detecting weak carcinogenic activity or reducing the duration of topical carcinogenesis bioassays.—J. Nat. Cancer Inst. 25: 1265–1277, 1960.

THE POTENTIAL carcinogenic hazard of chemical products that induce unforeseen biologic activity when introduced into the human environment emphasizes the current need for sensitive bioassay methods that require a minimum of time to detect carcinogenicity. The need is especially acute for methods of detecting weakly active materials whose biologic effects are induced by prolonged exposure, usually in combination with other agents. The pilot study herein reported was undertaken primarily to ascertain the effectiveness of limited preliminary treatment of mice with relatively large doses of benzo[*a*]pyrene as a device for amplifying and accelerating the neoplastic response to inherently weak carcinogens or to potent agents in high dilution.

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MATERIALS AND METHODS

Approximately 320 male and 390 female inbred SWR mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine. They were housed in transparent plastic cages and had free access to Purina chow and water. Each sex was randomly distributed to provide approximately 9 males or 11 females per cage. One cage of males and one of females were assigned for each combination of treatments employed. To identify the mice individually, their toes were clipped. Before the initial exposure, and periodically as needed, they were shaved in the interscapular region with electric clippers.

The experimental design was patterned to demonstrate: (*a*) the response of SWR mice to approximately 94 μg of benzo[*a*]pyrene in toluene solution, applied dropwise 3 times a week, to the shaved interscapular skin for 2, 4, or 8 weeks, or until a tumor appeared; (*b*) the effects of lifelong, thrice weekly applications of a 0.0075 cc drop of toluene solution containing either 0.75 or 3.75 μg of benzopyrene, 15 or 75 percent of a carcinogenic light creosote oil, or 5 percent of an industrial tar acid fraction; (*c*) the resultant biologic effects of summating, serially, each treatment listed in *a* with each in *b*. A lapse of 3 weeks was allowed between completion of the *a* series of primary treatments and initiation of the *b* series of secondary treatments, to insure the disappearance of fluorescence due to benzo[*a*]-pyrene applied in the first stage of the experiment.

For the primary treatments, commercial benzo[*a*]pyrene was prepared as a 1.25 percent solution in toluene on a weight-per-volume basis. Applications consisted of a single drop of the stock solution applied thrice weekly to the shaved interscapular skin with a tuberculin syringe and blunt hypodermic needle. The needle was calibrated to deliver approximately 133 drops per cc. Groups of mice were treated until a tumor was induced, for 8, 4, or 2 weeks, and were started on exposure in that order so that the various primary treatments might be terminated at about the same time. Control mice that received no primary carcinogenic treatment were merely shaved periodically.

For secondary treatments the materials used were: 1) toluene for control exposures; 2) two concentrations of benzo[*a*]pyrene, 0.01 and 0.05 percent in toluene, prepared on a weight-per-volume basis; 3) two concentrations of light creosote oil, 15 and 75 percent in toluene, prepared on a weight-per-weight basis; and 4) a solution of 5 percent tar acids in toluene (w/w). The light creosote oil was a crystal-free industrial mixture previously found carcinogenic though free of benzopyrene (*1*). The tar acids were an industrial acid fraction of high-temperature coal tar, principally phenol, cresols, and higher tar acids, which was previously shown to be noncarcinogenic even though it was a potent skin irritant (*2*). One drop of solution was applied thrice weekly to designated groups of mice, with a syringe and needle calibrated as indicated. Exposures were discontinued when a mouse developed a skin tumor 6 or more mm in diameter or became moribund.

Mice dying before the 20th week of the experiment were discarded, except in those groups in which a skin tumor was induced before that time. All survivors in a cage with a mouse that developed a tumor prior to the 20th week of the experiment were considered to have been adequately exposed, and data concerning these are included. All mice surviving 20 weeks of the experiment were considered as part of the experiment, regardless of the tumor response in their group.

RESULTS

Data for this experiment are summarized in tables 1 to 4.

Duration of Primary Treatment Period and Intensity of Carcinogenic Reaction

The dependence of the induced carcinogenic effect on the length of the initial carcinogenic exposure period is readily apparent in these tables. The effects of the different primary exposure periods *per se* can be approximated from the incidence of tumors (tables 1 and 3) and the time for tumor appearance (tables 2 and 4) of the mice whose secondary treatments were limited to toluene (group 1). The summation of those effects with that of subsequent exposures is apparent on comparison of the tumor incidence and rapidity of tumor appearance during the secondary exposure period for the toluene-treated mice (group 1) with that of the 0.01 or 0.05 percent benzopyrene-treated groups (2 and 3), the creosote-treated groups (4 and 5), and the group treated with the tar acid fraction (6). Within each group, the gradient in neoplastic response is determined in part by the length of time during which the primary applications of benzopyrene were made. An increase in duration of the initial benzopyrene exposure period is associated with an increase in tumor incidence and a reduction in time for tumor appearance, regardless of the nature of the secondary exposures (tables 1–4).

Four weeks of primary treatments with 1.25 percent benzopyrene provided a minimal, completely effective carcinogenic exposure period for the males: Epidermoid carcinomas developed after the primary treatments on 3 of 9 control mice treated subsequently with toluene (table 1, group 1). The first tumor on the most sensitive mouse was observed 25 weeks after completion of the "rest" period and initiation of the control treatments; the second mouse in order of susceptibility responded in the 36th week, while the last mouse to develop a tumor responded in the 63d week (table 2, group 1C). These observations—the time of tumor appearance on the two most sensitive mice in the experimental group (tables 2 and 4, cols. *a* and *b*), and the ratio of maximum induced-tumor incidence to the time required to reach that incidence (col. *c*)—represent criteria that have been consistently valid in our laboratory for estimating the relative carcinogenicity of pure chemical agents.

TABLE 1.—Incidence and types of skin tumors in carcinogen-sensitized SWR male mice

Weeks of primary applications 94 μg benzo[a]pyrene applied 3×/week		Secondary treatments*—0.0075 cc of solution containing	Initial No. of mice	Number with induced skin tumors during lifetime/No. effectively exposed	Histology of tumor at death	
Group	Weeks				Benign or regressed	Epidermoid carcinoma
1A	0	Toluene	9	0/9	0	0
1B	2		9	0/5	0	0
1C	4		9	3/9	0	3
1D	8		9	8/9	2	6
1E	†		9	9/9	2	7
2A	0	0.75 μg Benzo[a]pyrene in toluene	9	2/9	2	0
2B	2		9	3/8	2	1
2C	4		8	5/8	2	3
2D	8		9	9/9	1	8
2E	†		9	9/9	2	7
3A	0	3.75 μg Benzo[a]pyrene in toluene	9	6/8	2	4
3B	2		9	6/9	1	5
3C	4		8	7/8	1	6
3D	8		8	7/7	0	7
3E	†		8	8/8	0	8
4A	0	Creosote oil 15% in toluene	7	3/4	1	2
4B	2		7	4/5	1	3
4C	4		9	9/9	1	8
4D	8		9	6/6	1	5
4E	†		9	9/9	0	9
5A	0	Creosote oil 75% in toluene	8	5/6	5	0
5B	2		9	7/7	4	3
5C	4		7	7/7	0	7
5D	8		9	9/9	0	9
5E	†		8	8/8	1	7
6A	0	5% Tar acids in toluene	9	0/9	0	0
6B	2		9	0/8	0	0
6C	4		9	4/9	4	0
6D	8		8	6/7	2	4
6E	†		8	8/8	2	6

*Dropped on interscapular skin 3 times per week for life or until malignancy developed (*i.e.*, skin tumor with a diameter of 6 or more mm and a thickened, infiltrating base).

†Until a tumor 1 to 2 mm in diameter was induced.

Among the females, the minimal, completely effective exposure period was 2 weeks of primary treatments, resulting subsequently in benign papillomas on 2 mice and epidermoid carcinomas on 2 others, of 10 mice (in control group 1B, tables 3 and 4), after 32 to 71 weeks of secondary toluene applications

Tumors on the most sensitive mice of both sexes "primed" for 8 weeks or longer (groups D and E, tables 2 and 4) were observed during or before the first week for initiating secondary treatments (groups 5D, in table 2; 1D and 2D, in table 4). Eight weeks of pretreatment, under the experimental conditions employed, were obviously excessive for amplifying the carcinogenic effects of a subsequently applied agent for the most susceptible mice on experiment.

TABLE 2.—Skin-tumor incidence and tumor appearance time for carcinogen-sensitized SWR male mice

Primary treatment—1.25% benzo[a]pyrene in toluene 3×/week for:		Rest period (weeks)	Secondary treatment: Indicated solution applied 3×/week until death or tumor developed*											
			1 Toluene			2 0.01% Benzo[a]-pyrene in toluene			3 0.05% Benzo[a]-pyrene in toluene			4 15% Creosote oil in toluene			5 75% Creosote oil in toluene			6 5% Tar acids in toluene		
Group	Weeks		*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
A	0	3	—,	—,	0/112†	36,	55,	22/55	29,	38,	75/56	38,	55,	75/56	29,	30,	83/42	—,	—,	0/78†
B	2	3	—,	—,	0/58†	34,	41,	38/76	25,	27,	67/32	30,	30,	80/45	13,	16,	100/24	—,	—,	0/76†
C	4	3	25,	36,	33/63	18,	23,	62/56	14,	14,	88/28	6,	14,	100/40	3,	11,	100/24	27,	27,	44/74
D	8	3	0,	0,	89/32	0,	0,	100/30	0,	6,	100/27	0,	3,	100/24	−6,	−5,	100/9	0,	0,	88/46
			Tumor incidence and range in tumor appearance time due to primary treatment only																	
E	‡	3	8,	10,	100/14	6,	11,	100/14	9,	11,	100/16	6,	7,	100/13	7,	10,	100/16	7,	10,	100/14

*a. Weeks of secondary treatment preceding first tumor on most susceptible mouse in group.
*b. Weeks of treatment preceding tumor on next most susceptible mouse in group.
*c. Percent maximum tumor incidence / Weeks of secondary carcinogenic exposure.

†Experimental lifespan of oldest survivor in group.

‡Until a tumor 1 to 2 mm in diameter was induced. Secondary treatment in group E produced no significant difference in incidence of epidermoid carcinomas or lifespan, even though continued until tumors appeared grossly malignant.

TABLE 3.—Incidence and types of skin tumors in carcinogen-sensitized SWR female mice

Weeks of primary applications 94 μg benzo[a]pyrene applied 3×/week		Secondary treatments*—0.0075 cc of solution containing:	Initial No. of mice	Number with induced skin tumors during lifetime/No. effectively exposed	Histology of tumor at death	
Group	Weeks				Benign or regressed	Epidermoid carcinoma
1A	0	Toluene	11	0/10	0	0
1B	2	Toluene	11	4/10	2	2
1C	4	Toluene	11	6/10	3	3
1D	8	Toluene	11	8/11	2	6
1E	†	Toluene	11	11/11	0	11
2A	0	0.75 μg Benzo[a]pyrene in toluene	11	1/11	1	0
2B	2		11	2/8‡	0	2
2C	4		10	2/10‡	1	1
2D	8		11	9/11	1	8
2E	†		11	10/10	0	10
3A	0	3.75 μg Benzo[a]pyrene in toluene	11	9/10	5	4
3B	2		11	8/8	0	8
3C	4		9	7/9‡	1	6
3D	8		11	11/11	0	11
3E	†		11	8/8	0	8
4A	0	Creosote oil 15% in toluene	11	7/9	1	6
4B	2		11	11/11	3	8
4C	4		11	8/9‡	2	6
4D	8		11	9/9	2	7
4E	†		11	9/9	0	9
5A	0	Creosote oil 75% in toluene	8	7/8	2	5
5B	2		9	6/7‡	1	5
5C	4		12	12/12	0	12
5D	8		11	11/11	0	11
5E	†		11	11/11	0	11
6A	0	5% Tar acids in toluene	10	0/7	0	0
6B	2		11	1/10	1	0
6C	4		10	2/9	1	1
6D	8		11	10/10	6	4
6E	†		11	9/9	1	8

*Dropped on interscapular skin 3 times per week for life or until malignancy developed (*i.e.*, skin tumor with a diameter of 6 or more mm).

†Until a tumor 1 to 2 mm in diameter was induced.

‡An excessive number of premature deaths occurred in these groups after 20th week of experiment. *See* footnote 5 in text, p. 1272.

Dose of Secondary Carcinogenic Treatment and Intensity of Carcinogenic Reaction

During the secondary treatment periods, the greater carcinogenic response was elicited by the higher of the two dose levels for chronic applications of each carcinogen. Among the females that received 0.05 percent benzo[a]pyrene as secondary treatment (table 4, group 3A) the first tumor appeared after 36 weeks, whereas among those treated with a 0.01 percent solution the only tumor appeared after 49 weeks. At the

TABLE 4.—Skin-tumor incidence and tumor appearance time for carcinogen-sensitized SWR female mice

Primary treatment—1.25% benzo[a]pyrene in toluene 3×/week for:		Rest period (weeks)	Secondary treatment: Indicated solution applied 3×/week until death or tumor developed*					
			1 Toluene	2 0.01% benzo[a]-pyrene in toluene	3 0.05% benzo[a]-pyrene in toluene	4 15% Creosote oil in toluene	5 75% Creosote oil in toluene	6 5% Tar acids in toluene
Group	Weeks		a b c	a b c	a b c	a b c	a b c	a b c
A	0	3	—, —, 0/89	49, —, 9/49†	36, 43, 90/64	41, 45, 78/56	20, 20, 88/36	—, —, 0/92†
B	2	3	32, 60, 40/71	18, 56, 25‡/56	30, 32, 100/35	20, 24, 100/36	16, 20, 86‡/34	25, —, 10/25
C	4	3	13, 17, 60/63	18, 25, 20‡/25	18, 20, 78‡/25	12, 12, 89‡/32	6, 9, 100/25	18, 43, 22/43
D	8	3	0, 0, 73/40	−3, −2, 82/40	7, 8, 100/23	2, 3, 100/15	3, 3, 100/19	0, 0, 100/34
			Tumor incidence and range in tumor appearance time due to primary treatment only‡§					
E‡		3	8, 9, 100/16	8, 9, 100/14	12, 12, 100/15	8, 10, 100/13	9, 10, 100/16	11, 13, 100/16

*a. Weeks of secondary treatment preceding tumor appearance on most susceptible mouse in group.

*b. Weeks of treatment preceding tumor appearance on next most susceptible mouse in group.

*c. Percent of maximum tumor incidence / Weeks of secondary exposure until last tumor was induced.

†Experimental lifespan of oldest survivor in group.

‡Excessive number of deaths after 20th week. *See* footnote 5 in text, p. 1272.

§Until a tumor 1 to 2 mm in diameter was induced. Secondary treatment produced no significant difference in incidence of epidermoid carcinomas or lifespan in group E.

higher level a tumor incidence of 90 percent was obtained after 64 weeks. Similar effects are shown in tables 2 and 4 for the creosoted females and comparably exposed males.

None of the mice treated with a 5 percent solution of the tar acid mixture developed a skin tumor that might be attributed to that treatment alone or in part (group 6, tables 1–4). This observation supports a previous report on the absence of topical carcinogenic potency for the high temperature coal-tar acid fraction, either at higher and more toxic levels (*2*) or at the nonerosive dose level herein reported.

Intensity of Carcinogenic Reaction After Serial Applications of Primary and Secondary Carcinogenic Treatments

The responses of mice subjected to both primary and secondary courses of treatment indicate a distinct summation of sequential carcinogenic effects induced by each series. For each carcinogen, the pattern of response indicates an augmentation of tumor incidence and a reduction in time for tumor appearance, associated either with a longer primary treatment period or with a higher dose level of secondary treatment (tables 2 and 4, groups 2–5, B–D). In comparable groups where tumors developed on all effectively exposed mice, the differences in carcinogenic potencies, for the individual combinations of exposures, are reflected in the reduced time for tumor appearance on the most sensitive mice (table 2, groups 4C and D; 5B, C and D, *cf.* figures in cols. *a* and *b*). The same indication of differences in carcinogenic potency is shown in table 4, even in groups where a significant number of mice died after the 20th week of the experiment without tumors, and expected incidences of 100 percent were not achieved [5] (compare cols. *a* and *b*, groups 3B–D, also 4B–D). These data suggest again that the comparative response of the most sensitive mice within exposure groups is apparently a valid indication of relative carcinogenicity, obtained much earlier than the ratio of maximum tumor incidence to time required to develop that incidence (col. *c*, tables 2 and 4).

The absence of carcinogenicity for industrial coal-tar acids is confirmed by comparison of the carcinogen pretreated control mice with those similarly pretreated and subsequently subjected to applications of the tar acid solution (groups 1 and 6, tables 1–4). In fact, a slight but appreciable tumor-inhibiting effect is evidenced after tar acid applications, as indicated by the lower incidence of tumors among the 2- and 4-week carcinogen-primed females subsequently treated with the tar acid solution, in comparison with the toluene-treated controls (tables 3 and 4). Among the male mice a similar indication of tumor inhibition appears among the 4-week

[5] Unrelated to the type or dose of secondary carcinogenic exposure were some relatively poor lifespans, especially among the females in the indicated groups (tables 3 and 4). The premature deaths, and as a consequence, the comparatively low incidences of tumors in those groups emphasize the importance of healthy animals, free of parasites and chronic, mildly debilitating infections, particularly for long-term studies. However, even with these handicapped groups, the over-all pattern of reaction suggests a positive summation of carcinogenic effects, related either to the duration of pretreatment with the potent carcinogenic solution of benzopyrene or to the dose level of "test" material applied during the secondary course of treatment.

pretreated mice, when the benign tumors on 4 of 9 tar-acid-treated mice are compared with the epidermoid carcinomas on 3 of 9 mice whose secondary treatments consisted of toluene (table 1, groups 1 and 6, compare subgroup C). These differences, among the groups pretreated up to 4 weeks, were obliterated by longer periods of primary carcinogenic exposure, as shown by the reactions of the groups pretreated for 8 or more weeks.

DISCUSSION

The objective of this study was to determine whether pretreatment of the mouse skin with a known carcinogen would enhance the subsequent action of a weaker carcinogen at the same site, and would thereby indicate a summation of persistent biologic effects of the two agents. That objective was attained in finding a direct relationship between the induced carcinogenic response and the dose levels and total duration of effective, serial exposures [6] to both carcinogens.

Earlier work showed that a range of doses below 0.5 μg benzo[*a*]pyrene per skin application produced no infiltrating malignancy in highly inbred strains of mice despite lifelong repeated applications (*3*, *4*). The tumor response to those treatments was merely a low incidence of benign papillomas or carcinomas *in situ* on mice whose lifespans exceeded that of their controls and non-tumor-bearing cagemates. Those observations indicated the existence of threshold responses to topical carcinogens and suggested the now confirmed observation that the induction of papillomas in a fraction of an exposed mouse population is a reproducible threshold response to a trace environmental carcinogen—even in the absence of malignancies (*4*). The present data substantiate the preceding observations and indicate, in addition, an approach for enhancing such threshold responses, through the summation of sequential effects induced by calibrated exposures to a standardized potent agent and subsequent exposures to test materials of weaker potency.

The pertinence of the data to the design of a more efficient method for carcinogenicity bioassay requires consideration of at least three problems not studied definitively in this investigation: (*a*) the capacity of a tissue pretreated with one carcinogen to respond in accelerated fashion to another carcinogen, especially to one that is chemically, physically, or biologically dissimilar to it, (*b*) the applicability of the technique to exposure routes other than the percutaneous one, and (*c*) the feasibility of improving the efficiency and brevity of exposure necessary for an adequate subcarcinogenic primary effect. Fortunately, these questions can be considered in relation to data in the literature, principally in studies on the action of carcinogens and the process of carcinogenesis.

In reference to the first and second problems, one of the earliest studies

[6] The term "effective, serial exposures" is used to imply exposures that are neither of an intensity that exceeds the reactivity of the exposed tissue, nor subthreshold for eliciting a response after chronic applications.

on the biologic summation of carcinogenic effects was reported by Hieger, in a paper on the mechanism of carcinogenesis by chemical compounds (*5*). In it, he described the additive effects of consecutive applications of benzo[*a*]pyrene and dibenz[*a*,*h*]anthracene. Lavik, Rusch, and their coworkers later extended the observations of Hieger and found that the process of neoplasia initiated by any 1 of the 3 carcinogens, 7,12-dimethylbenz[*a*]anthracene (DMBA), benzopyrene, or 3-methylcholanthrene (MCA), could invariably be completed by the other two (*6*, *7*). The capacity of a tissue to summate residual carcinogenic effects after a lapse of 3 months between applications of methylcholanthrene was also reported by Lavik (*6*) and subsequently by others. In our laboratory, a similar state of carcinogen "sensitization" was found to persist for at least a year in mice treated with benzopyrene (*3*).

A summation of subcarcinogenic effects by dissimilar agents resulting in malignant growth was first demonstrated by Rous and associates when benign tumors, induced in rabbits' ears by the application of tar, were made invasive by the intravenous injection of the Shope papilloma virus (*8*, *9*). More recent data permit the inference that a similar phenomenon may be attained through the summation of carcinogenic effects of serial exposures to DMBA and orally administered acetylaminofluorine (AAF) (*10*), polycyclic carcinogens and croton oil (*11*), oral AAF and croton oil (*10*), β-radiation and MCA (*12*), urethan and X rays (*13*), urethan via many routes and croton oil (*14*, *15*), and coal tar and ultraviolet light (*16*, *17*). An acceleration in the process of carcinogenesis may thus be brought about through the summated effects of serial exposures to (*a*) the same or similar agents, (*b*) chemically dissimilar agents, and (*c*) mixtures of chemical and physical (radiation) or biologic (viral) agents, which affect tissues by different routes of exposures. It is apparent also that the residual effect of an adequate carcinogenic "priming" exposure is not specific for the "priming" agent. It can be summated by the affected tissue with other, later responses to diverse carcinogens, acting at dose levels and for periods that are above threshold in eliciting a response, yet subcarcinogenic by themselves.

With reference to the third problem, it is apparent in the present study that 2 to 8 weeks of primary treatments followed by a 3-week rest period are too time-consuming and expensive for routine application in a screening procedure. In this respect, the literature on the "two-stage-concept" of tumor initiation and promotion is highly significant. Historically, the investigations of MacKenzie and Rous (*9*), Rous and Kidd (*8*), and Berenblum (*18*) led to the formulation of this concept of tumor genesis: as a working hypothesis, it probably inspired investigations and advanced the understanding of carcinogenesis more than any other hypothesis to date. Although its basic assumptions of initiators, promoters, cocarcinogens, etc., are no longer considered tenable by some investigators (*3*, *15*, *19*, *20*), the studies that ensued suggest, in part, that a single skin application of MCA or DMBA may induce a preneoplastic state comparable with, or superior to, that obtained by 2 or more weeks of benzopyrene

treatments. The possibility of achieving an adequate "sensitizing" or "priming" exposure through a single application is thus strongly suggested. However, a quantitative study is obviously necessary to calibrate levels of preneoplastic, premalignant, and malignant responses that may be elicited by graded doses of those agents on the skin.

The fundamental assumption for tumor initiation made by proponents of the "two-stage concept of carcinogenesis" was of an instantaneous, irreversible transformation of normal cells to malignancy during the initial carcinogenic exposure. Since "initiation," the hypothetical instantaneous conversion of normal cells to malignancy, was presumably brought about as an all-or-none interaction between the carcinogen and a critical cell component, the dosage of carcinogenic exposure and persistence of the carcinogen at the site of application were usually disregarded. Similarly disregarded was the possibility of a physiologically irreparable state of tissue impairment or hormonal imbalance induced by the agent, to which the normal cells of the host react, in an ineffective, homeostatic response, by progressively less restrained cellular proliferation, with malignancy as the final host product (*1*, *3*). The neglect of a quantitative approach in studies that assumed the "two-stage" working hypothesis may thus account for a number of reported findings that appear irreconcilable or inconsistent with those herein described, among them that "the more application of carcinogen given, the fewer the tumors produced," (*21*), that there is no threshold dose of a carcinogen that can be considered harmless (*22*), and that tumors induced by so-called noncarcinogenic "promoters" or "cocarcinogens" are largely benign and show a high proportion of regressions, while "those induced by low concentrations of carcinogens usually appear malignant *de novo* or soon turn into malignancies" (*23*). Current indications, based on a quantitative experimental approach and a re-evaluation of older literature, confirm, on the contrary, the views that (*a*) multiple sequential changes are involved in an evolution, rather than an instantaneous production, of a state of malignancy (*1*), and (*b*) benign and malignant tumors are induced by agents heretofore considered noncarcinogenic promoters or cocarcinogens (*15*, *19*, *20*), simply because some agents formerly considered promoters are, in effect, inadequately tested weak carcinogens (*19*).

In regard to previously described "promoters" or cocarcinogens the careful retesting of such materials appears necessary to determine whether they are indeed weakly active carcinogens or simply solvents mistakenly classified as "promoters" after tests in which they were applied too soon after a potent and persistent "initiator." The persistence of potent "initiators," *i.e.*, polycyclic carcinogens, at the site of deposition on the skin has been demonstrated after periods up to 2 weeks (*3*, *5*). The possibility must therefore be considered of investigators having mistaken for a "promoting effect" the augmentation of a carcinogenic response to the "initiating" carcinogen obtained by redissolving superficially inactive deposits of carcinogen in a test substance applied prematurely, *i.e.*, before a 2-week period, after the initiating exposure (*24–26*). The removal of

residual or superficial, biologically inactive carcinogen from the site of exposure can be accomplished easily by swabbing (*3*) or elutriation (*27*) with an appropriate solvent before the application of a material to be bioassayed. Omission of this experimental detail may have been an additional factor that confounded the interpretation of studies on cocarcinogenesis in the past.

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Tumor Formation With Transplants of Spleen or Liver From Mice With Virus-Induced Leukemia [1, 2, 3]

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SUMMARY

Solid tumors were established from grafts of both the spleen and liver of a strain DBA/2 mouse with a virus-induced leukemia. The tumors, composed of the large, undifferentiated mononuclear cells characteristic of this virus leukemia, were serially transplantable to 100 percent of DBA/2 mice. Thus far, after 21 serial generations the tumors contain appreciable amounts of virus. The course of the disease was more rapid in mice implanted with local tumors than in those inoculated intraperitoneally with the virus. Tumor-bearing mice succumbed with generalized leukemia within approximately 35 days in contrast to those injected with virus, which had an average survival time of about 70 days. However, when transplantation to the randombred Swiss mice was attempted, tumors rarely developed.—J. Nat. Cancer Inst. 25: 1279–1289, 1960.

THE NEOPLASTIC character of a virus-induced leukemia in adult mice (*1*) was disputed because no solid tumors were formed. Consequently, it was of interest to determine whether local tumor growth could be obtained with leukemic tissues from the virus-infected mice. This has now been accomplished independently in our laboratory and in that of Buffett and Furth (*2*).

Our experience was similar to theirs in that initial attempts to produce local tumors by means of subcutaneous grafts of tissue from leukemic mice generally failed, though the generalized systemic leukemia developed with marked splenohepatomegaly. We later noted that transplantation appeared to be related to the stage of the disease in the donor animal. Successful grafting occurred only late in the disease when the livers and spleens were massively infiltrated with unequivocally leukemic cells which resembled those of reticulum-cell sarcomas. It was also observed

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[4] The technical assistance of Miss Violet Darchun, Mr. Gilbert Holland, and Miss Carol Mays is gratefully acknowledged.

that serial transplantation seemed to be further correlated to the selection of a susceptible strain of mice, in this case the inbred DBA/2.

Buffett and Furth are of the opinion that the tumor, which they have produced with livers from mice with the same virus-induced leukemia, resembles a nonviral reticulum-cell sarcoma. Whereas the tumors developed in our laboratory have some morphological resemblance to theirs, thus far we have not observed any that are free of virus. After 21 serial transfer generations, these tumors continue to contain significant amounts of virus.

This report is concerned with the histologic features and the virus content of these tumors after serial passage.

MATERIALS AND METHODS

Origin of tumors.—Ten strain DBA/2 mice, 6 to 8 weeks old, were inoculated intraperitoneally with virus. Nine of the mice died of leukemia. The 1 surviving animal was killed on the 78th postinoculation day and its spleen and liver were removed aseptically. Fragments, about 2 mm in size, were cut from each organ. Bilateral implants were made by trocar into groups of 10 mice of the same inbred strain. Within 30 days, all the mice in both groups developed tumors at the sites of implantation, as well as generalized leukemia. Both the spleens and the livers were involved in these animals. The spleen-derived tumor (ST) and the liver-derived tumor (LT) have since been carried in serial passage in DBA/2 mice.

Titrations.—The leukemic tissues (tumor and spleen) to be tested for virus content were removed aseptically and ground in a tissue grinder with a Teflon pestle. A 10 percent suspension was made with buffered Locke-Ringer's solution (*1*) and centrifuged at 3000 rpm for 10 minutes. The supernatant fluid was then removed, and serial tenfold dilutions were made. In some instances the supernatant fluid was put through a Selas 03 filter, impermeable to *Escherichia coli*, and the serial dilutions were carried out on the filtrate. The LD50 was calculated according to the method of Reed and Muench (*3*).

Animals.—Young adult DBA/2 mice of either sex approximately 6 to 8 weeks old were used for serial passage of the tumors. Whenever sufficient numbers of these mice were available, they were also used in titration tests to determine the virus content of tissues. Otherwise the titrations were done in Swiss mice similar in age to the DBA/2, as noted in the text.

RESULTS

In previous experiments subcutaneous transplants of spleen and of liver of either Swiss or DBA/2 mice, in the early phases of the virus-induced disease (7 to 30 days after inoculation of the agent), failed to

form local tumors, though generalized leukemia developed in most instances. Sometimes, however, the spleen in the subcutaneous site retained its integrity, as is illustrated in figure 1. In this figure, the cells in general are not as well preserved as they usually are in the leukemic spleen *in situ*.

In the present successful experiment, the transplants of leukemic spleen and liver resulted in tumor formation at the site of inoculation in 100 percent of the recipient DBA/2 mice.

Although the incubation period was approximately the same as that seen when filtrate was injected (*1*), the course of the disease was shorter. Generalized leukemia, as evidenced by the many immature cells in the blood, appeared within about 3 weeks. The mice survived for an average of 35 days after implantation of the tumor fragments, whereas the mice given injections of filtrate survived for about 70 days. Other than the difference in survival time, the pathological changes in the spleen, liver, and marrow in both groups of mice were comparable at the time of death.

The organs used for grafting had been heavily infiltrated with leukemic cells. Figure 2 shows the spleen from which one of the tumors (ST) originated. The leukemic cells are seen in large groups, replacing the splenic parenchyma. The liver from which the second tumor series (LT) was derived is shown in figure 3. The leukemic cells have infiltrated the sinusoids in amounts exceeding the mass of the hepatic cords.

Figure 4 shows the first passage of the spleen-derived tumor growing subcutaneously. The tissue is scarcely identifiable as spleen except for an occasional trabecula. In the first passage of the tumor developed from the liver graft a bile duct can be detected (fig. 5), although the parenchymal cells of the liver fragment have completely disappeared. Tumor masses, composed mainly of one cell type consistent with the characteristic leukemic cell found in the blood, liver, spleen, and bone marrow, have been formed.

After the first serial transfer there was no longer any trace of the organs of origin and both series of tumors appeared identical. The tumor cells were large and showed only slight variation in size (estimated 15–20 μ). The cells were round or polygonal and had relatively distinct boundaries. The nuclei occupying ½ to ⅔ of the cells were large. They were somewhat vesicular, round or oval, and occasionally slightly indented. One or 2 nucleoli were frequently seen. In some passages, an occasional binucleated or multinucleated giant cell reminiscent of the Reed-Sternberg cell of Hodgkin's disease was seen. The cytoplasm of tumor cells was amphophilic and moderate in amount.

In general, the tumor cells resembled those seen in the virus-induced primary lesion in that they consisted of a relatively uniform population of the large, immature, mononuclear cells. The pleomorphism of the tumor cells was slight as compared with that described by Buffett and Furth (*2*). Minor pleomorphism had also been observed in the leukemic cell population in the spleen, liver, and bone marrow of the animals inoculated intraperitoneally with cell-free filtrate in our routine passages. In those organs, however, the finding of a multinucleated giant cell was not

taken as conclusive evidence that it was a tumor cell, for a megakaryocyte cut tangentially might give such an appearance.

A mouse found dead 27 days after transplantation with the second serial passage of tumor is seen in figure 6 and is representative of the pathologic findings seen to date in the tumor-bearing animals. A section of one of the tumors after 12 transfer generations is shown in figure 7. The tumor cells are indistinguishable from those of the first passage and continue to take in 100 percent of the mice receiving the grafts. They are now in the 21st transfer generation.

Gross and microscopic findings comparable to those in animals which had died after they had received injections of cell-free filtrates were observed at autopsy of the dead, tumor-bearing mice. The spleens were enlarged from 2 to 25 times normal size. The larger spleens exhibited a violaceous appearance imparted by various combinations of three microscopic findings: congestion, extramedullary hematopoiesis, and foci of immature reticulum cells. In a few mice, hemorrhages recent or organizing were seen. In some cases, small pearly tumor nodules were present which microscopically proved to be composed entirely of leukemic cells.

The livers were slightly or markedly enlarged, the surfaces of the larger ones having a reticular appearance. This corresponded microscopically to marked infiltration with leukemic cells. In some cases, small clusters of tumor cells were seen. The smaller livers showed prominent Kupffer cells, extramedullary hematopoiesis, and some infiltration with immature reticulum cells.

The sternal bone marrow showed foci of leukemic cells, but the lymph nodes, though slightly enlarged, microscopically showed only nonspecific hyperplasia without leukemic involvement. Other organs, including the thymus, were unremarkable both grossly and microscopically.

Periodically, throughout these studies, the spleens and tumors were titered at the time of transfer to determine the virus content of each of these tissues. Whenever possible the titrations were carried out in DBA/2 mice, since we find these inbred animals more uniformly susceptible to the agent. When these were unavailable, Swiss mice were used. The comparative titers of the virus in the spleens and tumors from mice bearing the serial transplants of the leukemic tumors are given in table 1. The titers obtained from the tissues of the spleen-derived (ST) and of the liver-derived (LT) passages are comparable. Thus far, there has been virus in all the tumors tested, and attempts to free the tumors of virus have failed.

When these tumors were grafted into the randombred Swiss mice, generalized leukemia developed in most instances, but growth of the tumor occurred only rarely. These occasional tumors were not readily transplantable to other Swiss mice. At best, it was possible to pass them for 3 generations with a take of between 10 and 30 percent. After the third passage only the generalized leukemia resulted from the graft.

In attempts to repeat the experiment which led to the development of the solid tumors, it was found that successful takes usually occurred when

TABLE 1.—Titer of virus in spleen and tumor from mice bearing tumors originating from leukemic spleen or liver

Generation No.	Mouse strain	Titer*	
		Spleen	Tumor
1†	DBA/2	4.0	3.6
2†	Swiss	3.2	3.8
		3.0‡	1.5‡
		3.3	2.0
3†	Swiss	3.1‡	1.7‡
5†	Swiss	3.5	4.0
5§	DBA/2	3.8	3.0
7§	DBA/2	3.4‡	—
8†	Swiss	1.5	1.2
11†	DBA/2	4.0	2.8
11§	Swiss	4.0	2.3
13†	DBA/2	3.5	2.0
17†	DBA/2	>4.0	>4.0
21§	Swiss	2.6	2.2

*Expressed as the reciprocal of the LD50.
†Spleen-derived tumor.
‡Filtrate-tested.
§Liver-derived tumor.

DBA/2 liver or spleen extensively infiltrated with leukemic cells was grafted into mice of the same inbred strain. The tumors that arose in these confirmatory studies were identified histologically, but were not carried further in serial passage. In similar duplicate experiments with the leukemic organs of the Swiss mice, transplants to mice of the same strain resulted in generalized leukemia, but solid tumor rarely formed at the site of the graft.

Immunological studies of the effect *in vitro* of virus antibody on tumor-cell suspensions and on the growth of tumors in mice vaccinated against the virus are in progress.

DISCUSSION

Since DBA/2 mice rarely develop spontaneous leukemia (*4*), it is unlikely that the leukemia of the original donor mouse was of spontaneous origin. The mouse in question was 6 to 8 weeks old when it was inoculated with virus and it was killed 78 days later to provide spleen and liver for the grafting experiment.

Earlier experiments of the same type with Swiss mice had not yielded results as significant. It was only when the implantation trials were made in the inbred DBA/2 mice that success was achieved. In these cases, massively infiltrated leukemic livers and spleens from this strain of mice gave positive results. When trials were made with mouse organs which histologically showed signs of disease but were not yet frankly neoplastic, only generalized leukemia resulted.

Richter and McDowell (*5*) noted that several thousand cells were required to transmit leukemia to their inbred mice, while Furth and Kahn (*6*) were successful with as few as one cell, provided this cell was malignant and capable of multiplying. Apparently the number of cells necessary for transmission varied with the strain of leukemia studied and the route of inoculation. From the evidence on hand, it would appear that there is a relation between transplantability by the subcutaneous route and both the number and the degree of immaturity of the neoplastic cells grafted. When a sufficient number of the transplanted cells are the large, immature, neoplastic ones, tumors generally grow. Cells that are not in this stage are probably incapable of reproducing, but are good sources of virus. The neoplastic cells that are so immature as to approach anaplasia, on the other hand, have acquired the added ability to multiply. That they produce virus as well has been shown in electron micrographs of the tumors (*7*). The relation of the virus to the tumor cell, while not clear at this stage of our knowledge, appears to be a vital one.

Whether the state of the neoplastic cells at the time of transplantation and/or their actual number is the determining factor awaits further study. So far, it has been impossible to distinguish, histologically, immature reticulum cells, taken late in the disease, that proved to have the ability to form local tumors, from those that were taken a shorter time after inoculation of the leukemia virus but failed to form local tumors.

Buffett and Furth have offered a hypothesis to explain the successful grafting of leukemic liver in Swiss mice in their laboratory. These investigators have concluded that the tumors resembled autonomous reticulum-cell sarcomas since they exhibited a pleomorphism not noted in the leukemic organs of mice inoculated with virus filtrate. However, they leave open the possibility that the virus may still be necessary for the growth of the tumor since transplantability seemed to be correlated, as in our work, with the generalized leukemia of this virus-induced disease. Successful passage occurred when the donor mice had the characteristic splenohepatomegaly, but a test for the presence of virus was not made.

If, however, these tumors prove to be virus-free, it will be of interest to examine them further in the light of the recent findings of Prince (*8*). He has reported that the serial passage of "noninfective" Rous sarcoma which did not produce detectable virus resulted in the return of virus-containing tumors. He concluded that the "noninfective tumors" were a "consequence of a nonproductive form of cell-virus integration."

Whereas the cells in the tumor described here are more uniform than those observed by Buffett and Furth, some pleomorphism such as they described is occasionally observed. This was seen not only in the tumor but also in the spleen and liver of both tumor-bearing mice and mice that had received cell-free filtrate, which indicates that this was not a primary feature of the tumor. Whether or not this histologic difference is because Buffett and Furth grafted the leukemic tissue in irradiated Swiss mice and we used nonirradiated DBA/2 mice remains to be determined.

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PLATE 165

All tissues fixed in Vandergrifft's solution and sections from them stained with hematoxylin and eosin.

FIGURE 1.—Subcutaneous fragment of spleen from a leukemic DBA/2 mouse. The spleen used for grafting had been removed from a mouse of the same strain 7 days after inoculation of virus. It had survived at the trocar site without tumor formation for 73 days. *Note* capsule and megakaryocyte in *upper-right corner*. × 340

FIGURE 2.—Leukemic spleen 78 days after inoculation of virus. Subcutaneous implants of this organ resulted in tumor formation. Pale, leukemic cells can be seen mainly in *upper-left corner*. Cells with dark nuclei are erythrogenic. × 340

FIGURE 3.—Leukemic liver from same mouse. The liver-passage tumor originated from this organ. There are vast numbers of leukemic cells in sinusoids. × 340

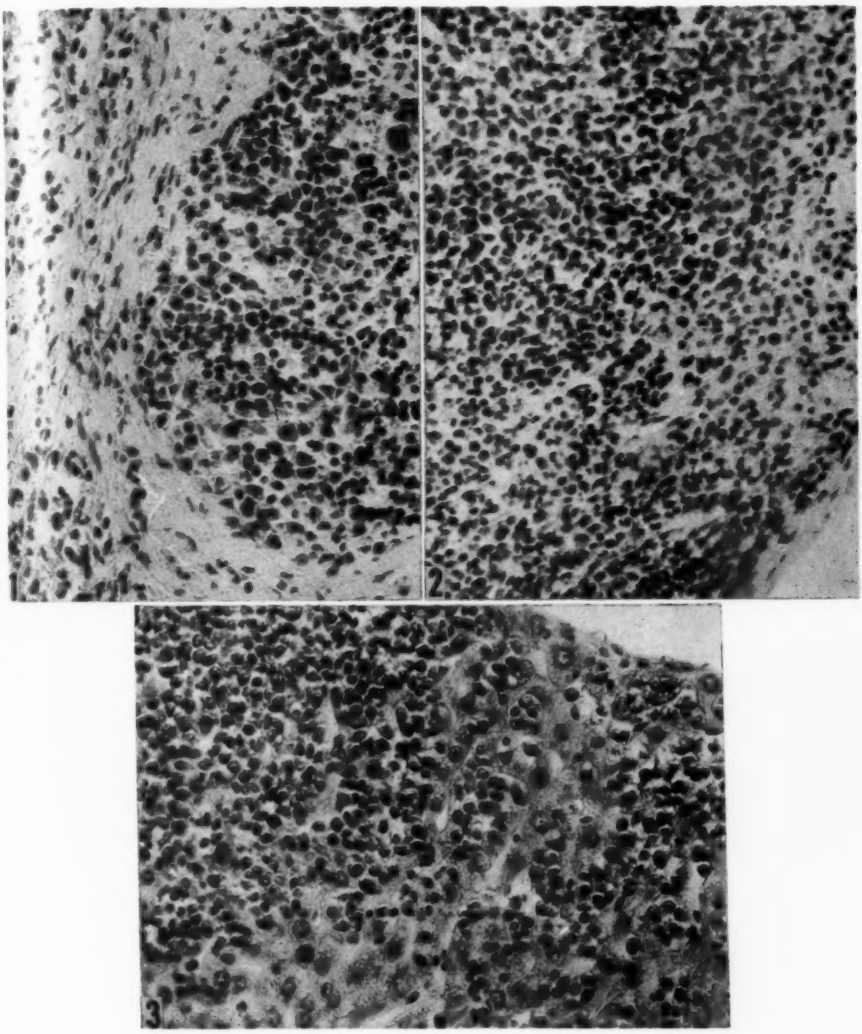


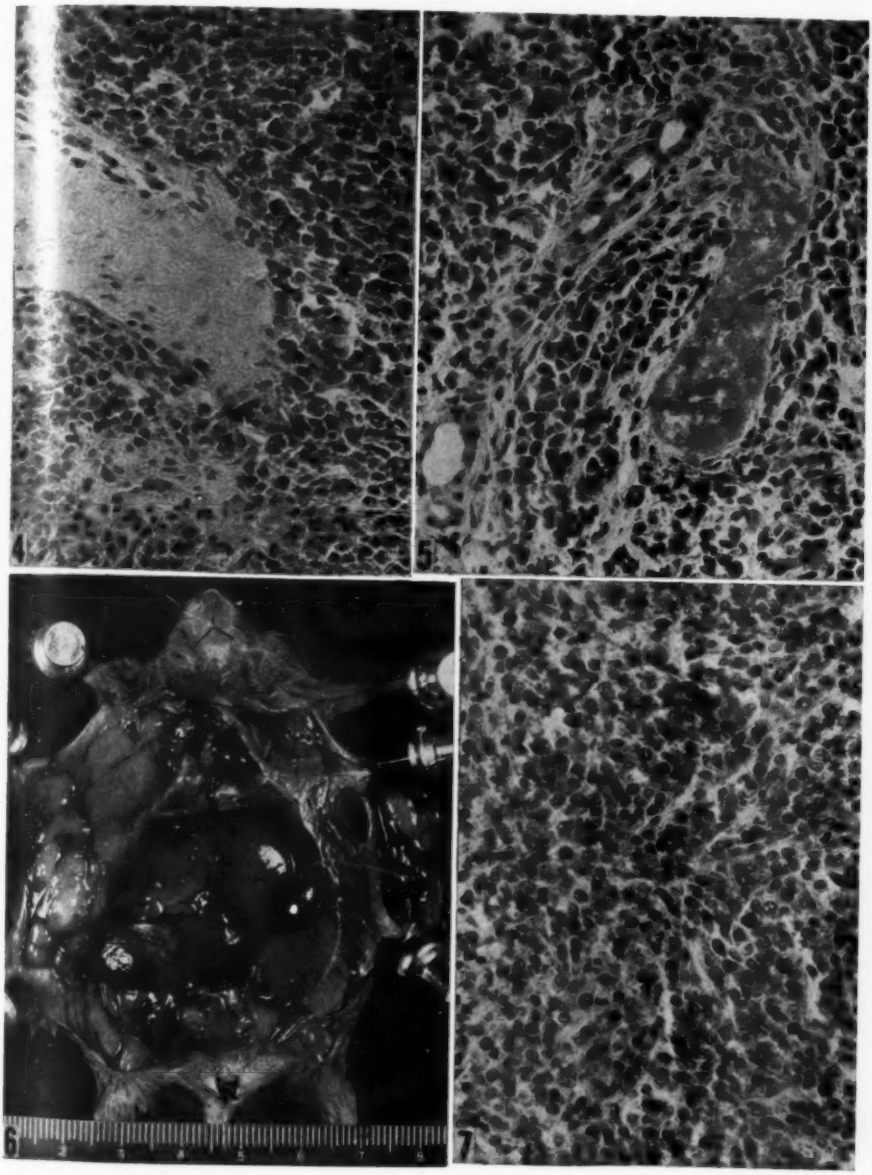
PLATE 166

FIGURE 4.—Tumor which arose from a subcutaneous transplant of the spleen shown in figure 2. *Note* necrotic trabecula. × 340

FIGURE 5.—Tumor which was derived from the liver shown in figure 3. A bile duct and a thrombosed portal vein branch are visible. × 340

FIGURE 6.—Bilateral subcutaneous tumors at sites of 2 mm transplants in DBA/2 mouse. This animal died 27 days post transplantation. *Note* size of tumors and marked hepatosplenomegaly.

FIGURE 7.—Tumor after 12 serial passages. Resemblance to a reticulum-cell sarcoma is marked. × 340



Deoxyribonucleic Acid Values (Feulgen Microspectrophotometry) in Epithelium of Human Ectocervix, Normal and Cancerous [1]

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SUMMARY

Absorption measurements of Feulgen-stained nuclei have been made on squamous epithelia in the human cervix uteri in precancerous and cancerous conditions. The scatter of values for the prepuberal and for 3 out of 8 postpuberal cervical epithelial histograms was fairly symmetrical and shaped like a wide variety of somatic tissues, including skin. In the remaining 5 postpuberal specimens there was a small group of nuclei at least double that of the mean for the tissue. In all the postpuberal specimens the mitotic rate was of the order of 1 : 10,000 cells. The scatter of values in the precancerous and cancerous specimens is characteristically broad, but broader in the so-called metaplastic lesion than in frank carcinoma. In the epithelia of otherwise normal cervices there were a few cells with high deoxyribonucleic acid values in the range of known values for cancer stem lines; the significance of their presence is discussed.—J. Nat. Cancer Inst. 25 : 1291–1301, 1960.

THERE IS ample evidence that cell nuclei of many neoplasms, including those of the squamous carcinoma group, are approximately tetraploid and frequently large. The concept of a stem line in the growth of cancer cells, as promoted by Hauschka *et al.* (*1*) and Makino (*2*), has been reinforced by microspectrophotometric methods on Feulgen-stained preparations (*3*, *4*) and by the chromosome counts of Manna (*5*). Apart from the origin of cells of prospective carcinomatous tendency in the tissue, the stem line with high deoxyribonucleic acid (DNA) value may be established early in carcinogenesis, and occasional large nuclei may be present in otherwise normal epithelia, where they may lie latent or be overlooked by usual methods of histologic examination. The chances of revealing such cells in cervical epithelium, particularly in small numbers, may be enhanced by an analysis of a histogram constructed for DNA values. In the histogram, attention may be directed to the incidence of those nuclei with high DNA value. Values about twice the mean for the tissue as a whole appear to be of particular interest (*3*).

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[2] Research student, Watson Munro Fund, University of Sydney.

[3] We wish to thank Professor K. W. Cleland, Department of Histology, University of Sydney, for his generosity in allowing us to use the Lison histophotometer, and for his helpful advice.

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MATERIALS AND METHODS

Specimens were obtained from biopsy, operation, and autopsy material. A small piece of epithelium was dissected with as little subepithelial tissue as possible and placed in 1 percent trypsin at 37° C for 30 minutes. This piece was then squashed on a slide with a coverslip by moderate finger pressure, and a smear was made to distribute the cells as widely as possible. The smear was then air-dried at 37° C and fixed in alcohol-acetic acid mixture for 2 hours. Control smears were prepared from guinea-pig epididymal sperm, and the slides of this material were treated back-to-back with all samples from the cervix.

The treatment of the specimen after removal varied in two samples to allow a more precise determination of the site of the cells' origin. In the first epithelium specimen a horizontal split was made by microdissection in a plane analogous with the stratum granulosum of the skin. Both superficial and deep moieties were subsequently submitted to trypsinization. In the second specimen a slice 3 mm wide was cut from the cervix as a radial strip from the junction with the vagina to the junction with the columnar epithelium of the cervical canal. Five sections were then divided off along the length of the strip so that the histogram of nuclei near the squamo-columnar junction could be compared with that from the nuclei nearer the vaginal end.

Mitotic counts of the full thickness of all epithelium specimens, based on the number of metaphases per 10,000 cells, were made on subjacent pieces taken for paraffin embedding and sectioning. The Feulgen stain was used according to the description of Darlington (*6*) at a *p*H of 1.6. The caprice of the stain reported in the literature has been experienced, and methods were devised to standardize the procedure as far as possible. Optimum staining was obtained after 12 minutes of hydrolysis. Absorption of reconstituted fuchsin was measured in the Lison histophotometer (*7*) modified to permit the projection of the image so that a drawing of a nucleus could be made for subsequent determination of the area by planimetry. The 550 mμ line of a high-pressure arc lamp was isolated by means of glass filters, Ilford 605, and the projected image sampled on a photomultiplier tube at an aperture corresponding to an area of 2 μ in the object plane. Since the data required were for use only relatively from cell to cell, no attempt was made to resolve the readings in terms of the extinction coefficient.

Six extinction readings were made arbitrarily from within the nucleus and 6 without the corresponding cell; these extinction values were averaged and subtracted. This value was then multiplied by the surface area of the nucleus, determined by planimetric measurements of the drawing made from the projected image. Error in planimetric measurements increased rapidly with drawings less than about 2 cm in diameter, so that a suitable objective was used in the microscope to insure that the projected image was about 4 cm in diameter. In this way, readings from the same drawing showed a variation no more than 3 percent. The combined

area and absorption were used as the arbitrary value of the nuclear content of reconstituted dyestuff, and histograms were constructed from it. Except in 2 instances, 50 cells from each specimen were measured in this way.

There is much published evidence as to the empiricism and hazards of Feulgen microspectrophotometry (*8–11*). In this study due attention was paid to minimizing the more obvious sources of error, and wherever possible frequent checks of apparatus and material were made. Confirmation of the obedience of the system to Lambert's Law was occasionally possible when measurements of two overlying sperm heads gave precisely twice the expected absorption.

RESULTS

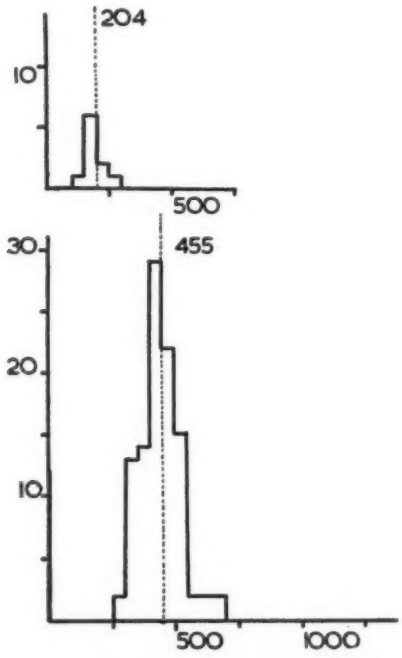
Results are presented in histogram as well as tabular form, since it is generally conceded that the mean value is no true representation of the spread of values and that such spread is of undoubted biological importance, particularly from the viewpoint adopted in this study. The results are divided into two groups: (*a*) from the cervices of normal histologic appearance, subdivided into those from prepuberal and postpuberal specimens; (*b*) from cases of pathologic appearance, designated metaplasia and carcinoma, respectively.

TABLE 1.—Mean values of DNA by Feulgen microspectrophotometry in *arbitrary* units and their associated standard deviations (SD) in cells of the squamous epithelium of the cervix uteri in classes

Case	Number	Mean	SD	% Cases >50% above Mean = 3N	% Cases >100% above Mean = 4N
			1.	Normal	
				(*a*) Prepuberal	
A	50	218	27	0	0
B	101	455	80	0	0
C	50	495	73	0	0
				(*b*) Postpuberal	
D	50	501	242	14	2
E	50	519	244	12	2
F	50	405	109	6	0
G	78	379	133	8	4
H	50	492	133	4	2
I	50	457	65	0	0
J	249	461	183	8	3
K	199	423	122	4	2
			2.	Precancerous	
L	100	928	952	14	11
			3.	Cancerous	
M	49	1105	594	Not calculated	

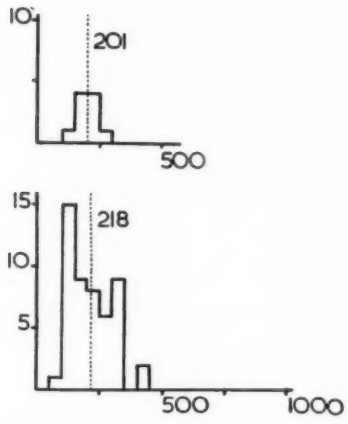
Normal Histology

In the histograms from prepuberal source material, of which text-figure 1 is representative, the distribution is of typical form, characteristic of many tissues. The mean is of the order of twice that of the accompanying guinea-pig sperm histogram. The distribution about the mean is fairly symmetrical, and there are no values greater than 50 percent above the mean. One of the three specimens examined had a mean little different from that of the associate guinea-pig sperm sample (text-fig. 2).

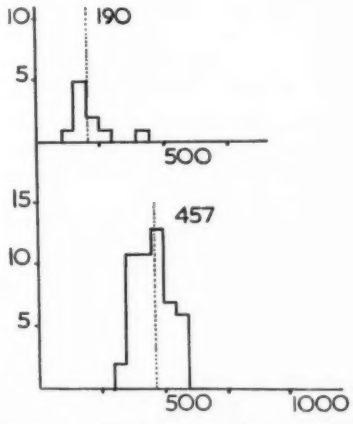


TEXT-FIGURE 1.—Histogram of DNA values by Feulgen microspectrophotometry of cells from epithelium of cervix uteri of 6-year-old girl. Accompanying smaller histogram in this and succeeding histograms is constructed from DNA values obtained simultaneously from guinea-pig sperm for use as reference point.

Of the 8 adult specimens, 2 groups may be recognized. Text-figures 3 and 4 are representative. In the first group, comprising 1 specimen, the distribution was compact and symmetrical about the mean, and there were no cells outside values 50 percent above the mean. In the second group, represented by text-figure 4, comprising the remaining 7 cervices, there was an average of 8 percent of the nuclear sample with DNA values over the 50 percent parameter, with a range of 4 to 14 percent, and of these 7, 5 samples had an average of over 2 percent of



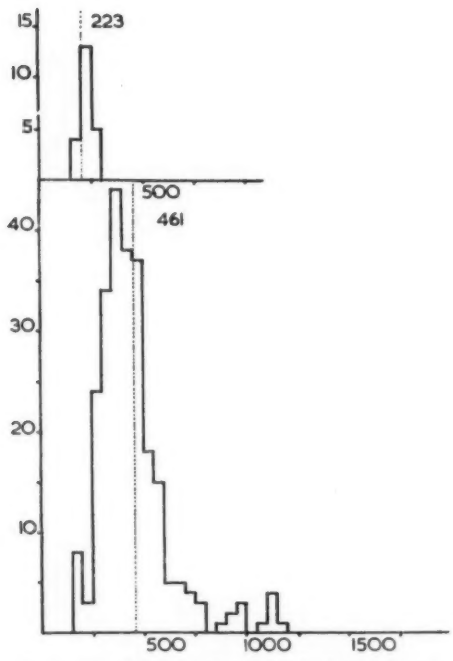
TEXT-FIGURE 2.—Histogram of DNA values by Feulgen microspectrophotometry of cells from epithelium of cervix uteri of 3-year-old girl. Mean differs little from that of accompanying sperm sample.



TEXT-FIGURE 3.—Histogram of DNA values by Feulgen microspectrophotometry of cells from epithelium of cervix uteri of parous adult. Distribution is symmetrical about the mean.

nuclei equal to or greater than 100 percent above the mean. In 2 cases, isolated nuclei had values up to 3 times that of the mean.

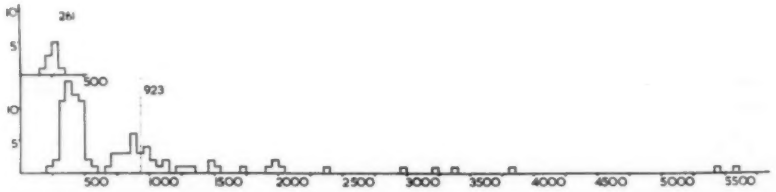
The two specimens, in which the sampling from the epithelium was made with more anatomic and histologic precision, showed in the nuclear content of DNA no significant differences in the shape of the histogram, as contrasted with that constructed with nuclei of more random sampling. In all specimens in the adult group the mitotic count was of the order of one metaphase per 10,000 cells.



TEXT-FIGURE 4.—Histogram of DNA values by Feulgen microspectrophotometry of cells from epithelium of cervix uteri of parous adult. In contrast with preceding figure, histogram has "tail" of values about 4N parameter.

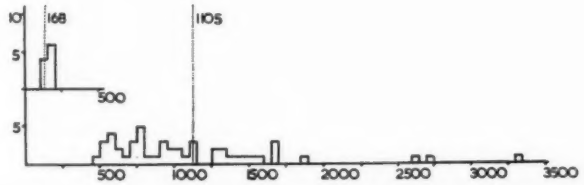
Abnormal Histology

Two specimens were analyzed. The first was taken from a so-called healing transformation zone which, on histologic examination, showed the epithelium to contain large numbers of irregularly shaped nuclei, multinucleated cells, and keratin in the more superficial layers (figs. 1 and 2). Such an appearance is loosely termed "metaplasia" and is widely regarded as a precancerous condition. Text-figure 5 shows relatively large spread of DNA values with classes up to ten times that of the mean.



TEXT-FIGURE 5.—Histogram of DNA values by Feulgen microspectrophotometry of cells from epithelium of cervix uteri of parous adult taken from portion of area of "metaplasia" of cervical squamous epithelium. Spread of values is very great even by contrast with text-figure 6.

The second specimen was from a Stage-II squamous carcinoma of the cervix of typical histopathologic pattern. The histogram presents a wide spread of values, though not as extensive as in the first specimen (text-fig. 6).



TEXT-FIGURE 6.—Histogram of DNA values by Feulgen microspectrophotometry of cells from epithelium of cervix uteri of parous adult taken from portion of invasive squamous carcinoma.

DISCUSSION

In general, the histograms of DNA values presented here are in agreement with those published for a variety of other normal and cancerous tissues with regard to the spread of values (*11–14*). There is, however, a difference with respect to the spread of values in certain normal adult cervices. In an analogous tissue such as skin epithelium, a representative histogram of which is figured by Leuchtenberger *et al.* (*15*), there are no values of DNA expressed in arbitrary units greater than 50 percent above the mean in 120 cells examined. This contrasts with an average of 8 percent above this parameter in the cervical epithelium in 7 out of 8 specimens, and in 5 of these specimens examined, an average of 2 percent above that parameter 100 percent greater than the mean. If there is sanction in the recent practice in assigning the diploid number to the average DNA value of a tissue, especially if this is about double the value of spermatozoa of a species with a chromosomal count roughly similar, then in broad terms there exists in the majority of histologically normal cervical epithelia examined a small but constant proportion of cells about the tetraploid number and above. This is in accord with the experience of Atkin and Richards (*3*). A spread of greater than twice the mean is also figured in the report by Mellors *et al.* (*13*) for normal cervical cells.

This spread in the histogram toward higher values in the "tail" may have a second explanation invoked by Swift (*8*), Leuchtenberger *et al.* (*12*), and Richards, Walker, and Deeley (*16*). These authors ascribe the intermediate values up to twice the mean to the measurement of cells in varying stages of synthesis up to double the amount of DNA in the resting cell during late interphase. An idealized histogram of this sort is figured by Atkin and Richards (*3*). However, in all specimens of our normal postpuberal material, the mitotic rate at one spindle per 10,000 cells was not greatly altered from figures of the general order of epithelial tissue.

Despite the obvious defects in straight counts of such spindles without pretreatment with colchicine, wherein all mitoses other than those preceding the precise time of fixation are overlooked, it is considered that this rate is sufficiently low to preclude the suggestion of an abnormally active state within the epithelium concerned. An analogous situation is reported in the wide scatter of DNA values among the nuclei of tissue-culture cells obtained by Kleinfeld and Chu (*14*). They noted the low mitotic activity of the culture at the time, inferring that the cause of the scatter was to be sought outside this factor.

There is insufficient evidence for the extent of the occurrence of haploid values as indicated by the low mean of DNA values in one of the histograms from a 3-year-old. The observation is recorded in passing.

In the group of abnormal cervices, extended spread of the histogram is in accord with the findings for a wide variety of neoplasms, both in the cervix and elsewhere (*3, 12, 13*). In the specimen labeled "metaplasia," increase of mitotic rate undoubtedly contributed *per se* to some of the scatter, but the majority of very high values derive from relatively large nuclei, some of which are multilobed and from actual multinucleated cells (figs. 1 and 2). The histologic appearance of the specimen showed none of the criteria of malignancy either *in situ* or invasive, yet the values of DNA are as high as and higher than those depicted in the frank squamous carcinoma. This stresses the obvious behavioral ingredient in the diagnosis of malignant neoplasia. The histogram of the squamous carcinoma is without a defined mode, which renders the assignment of a stem line difficult (*3*).

The general conformity of the distribution of the DNA values and the similarity in the standard deviations to those of others reported in the literature seem to confer no particular advantage on the method of multiple readings within one nucleus, possible in a squash technique. It may well be that a more faithful reproduction of the average absorption for the whole nucleus is countered by a greater error in reproducibility of the determinations of area by planimetry.

In conclusion, attention is drawn to the contrast between the histograms of the prepuberal cervix and those of the majority of adult cervices. If it is conceded that the various errors known to be associated with Feulgen microspectrophotometry do not vitiate the significance of the histogram and that the presence of cells of values greater than 100 percent above the mean is not associated with a demonstrable increase in mitotic rate, it is clear from this study that there reside often in the adult cervix a few cells which bear approximately double the mean DNA value. Note that this value is of the order of that exhibited in some cervical cancer stem lines. A successful search for isolated cells of this type among the normal population of cervical squamous epithelium, with standard histologic procedures, will be reported elsewhere.

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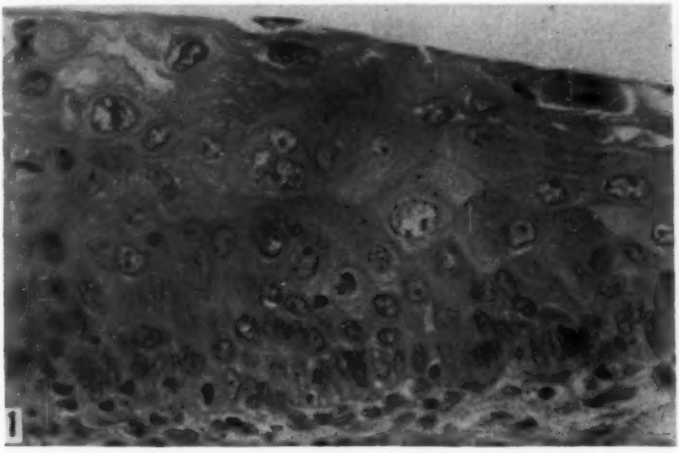
PLATE 167

FIGURE 1.—Portion of epithelium of ectocervix showing multinucleate and binucleate cells. Paraffin section, hematoxylin-eosin staining, 8μ. × 450

FIGURE 2.—Portion of same epithelium to show 2 cells, 1 with enlarged nucleus and the other with 5 nuclei. × 1,500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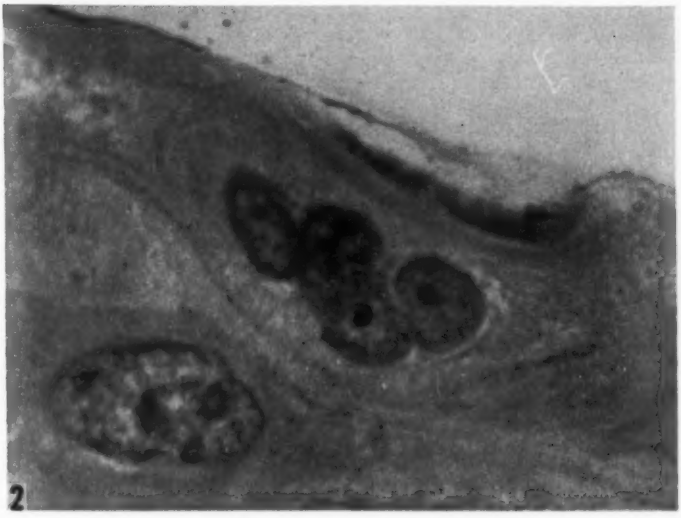
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Structural Differences Produced in Mammalian Cells by Changes in Their Environment [1]

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SUMMARY

Epidermal cells assume a different form and internal structure when they are cultured in tissue-culture medium containing protein or in medium without protein. They grow as flattened sheets of cells on the flask walls in a medium containing protein and in free-floating, rounded clumps in medium without protein. If they are cultured in protein-free medium in the presence of a Millipore filter, they attach themselves to it and grow in flattened sheets just as they do in medium containing protein. Cells grown in protein medium produce characteristic lamellar bodies which appear to be derived from mitochondria. The Golgi complex is highly developed in epidermal cells grown in any of the tissue-culture media in contrast to its vestigial nature in epidermal cells *in situ*. This is consistent with a possible osmoregulatory function, since the cells *in vitro* are in a water-rich tissue-culture medium. Cells growing in suspension exhibit an ability to reduce osmium tetroxide on postosmication of their mitochondria, which does not occur when they are grown in flat sheets. This is independent of the medium in which they grow. It is postulated that the surface configuration of the cell may influence the chemistry of its mitochondria.—J. Nat. Cancer Inst. 25: 1303–1323, 1960.

DIFFERENCES IN ability to promote growth of cells in tissue cultures exist among various media. It was thought by the authors that this difference might be reflected in the visible, fine structure of cells grown therein. A medium has been developed by Evans *et al.* (*1–3*), which was originally used for cultivation of Earle's strain L cells (*4*, *5*). When normal human epidermal cells are grown in this medium, some proliferation is obtained, but differences appear when the pattern of growth is compared to that obtained with other media. Human epithelial cells will form sheets, which adhere to the floor of the glass culture flask, when the cells are grown in a stock medium of 30 percent human serum and 20 percent chick-embryo extract in an isotonic solution of salts or in the chemically defined medium NCTC 109, developed for strain L cells, plus 10 percent added human serum. However, if the same strain

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of cells is grown in the synthetic medium alone, the cells will not adhere to the flask, but will grow as a suspension of single, rounded cells or small, round clumps. If a strip of Millipore filter is placed in the culture flask containing the synthetic medium, the epithelial cells will grow as a sheet of cells on this filter.

The present investigation was undertaken to ascertain any differences in the electron microscopic appearance of the cell structure of human skin cells that might be correlated with the differences in patterns of growth which occur under the influence of different physical and chemical environments.

MATERIALS AND METHODS

Two kinds of tissue-culture cells grown in three different media were used in these experiments: NCTC strain 929, line of strain L cells (*4*, *5*), and normal human skin epithelial cells, NCTC strain 2414 (*6*, *7*), from a 52-year-old white male. These cell types were grown in: 1) stock medium containing 20 percent filtered chick-embryo extract, Earle's balanced saline, and 30 percent serum, the serum being pooled horse serum for the strain L cells and pooled human serum for the human skin cells; 2) a chemically defined medium in which each constituent is known or controlled, as developed for strain L cells (*1–3*); and 3) chemically defined medium plus 10 percent serum, the serum being horse serum for the strain L cells and human serum for the human skin epithelial cells.

The cells grown in chemically defined medium were cultured either in sheets on a Millipore filter, which is chemically inert, or in sheets attached to the floor of the flask, or in suspension. Fixation was carried out for 15 minutes in either Dalton's (*8*) or Palade's (*9*) fixative. Cells grown in sheets on the floor of the flask were fixed, after decantation of the medium, by the addition of the fixative to the flask. For fixation of cells grown in suspension, the cells were first centrifuged, then the medium was decanted, and the fixative was added to the pellet. Cells grown on the filter were fixed by the filter being placed with its attached cells into the fixatives. Cells fixed in the pellet were washed 15 minutes in several changes of isotonic saline and then embedded by being mixed with an equal volume of a solution of 2 percent agar and 1 percent formol in water. After this cooled and set, the mass was cut in appropriately small pieces and dehydrated through a series of ethanol solutions. A second embedding was then carried out in a mixture of 1 part methyl to 9 parts *n*-butyl methacrylate to which was added 0.25 percent benzoyl peroxide, with polymerization at 60° C. Cells fixed on the side of the flask were washed in place and then scraped off, centrifuged into a pellet, the pellet embedded in agar, and subsequently treated as previously. The cells fixed on the filter were simply dehydrated and embedded in methacrylate, without being embedded in agar.

The intracellular location of certain reducing chemicals can be demonstrated by the method of postosmication described by Dalton and Felix (*10*). For this procedure, the cells were fixed in Palade's fixative, washed in tap water, and treated with an unbuffered 2 percent aqueous solution of osmic acid for 24 hours at 37° C. Tap water rather than distilled water was used because the chlorine content of tap water provides the proper oxidizing action to make the reducing materials available (Dalton: personal communication). Dehydration and embedding were the same as for the other cells.

Sections were cut with the Porter-Blum microtome set at 250 to 400 A and photographs taken with the RCA EMU-2C electron microscope.

OBSERVATIONS

The cells grown in stock medium and in chemically defined medium plus serum were indistinguishable and therefore can be considered together as cells grown on protein-containing medium. The cells grown in chemically defined medium (protein-free) were grown either in suspension, or as sheets of cells on the glass floor of the flask, or on Millipore filters. We then have three conditions of growth: 1) cells grown in sheets in protein medium; 2) cells grown in sheets in protein-free medium; and 3) cells grown in suspension in protein-free medium. The preceding categories are to be considered from three different aspects: (*a*) microscopic morphology and fine structure related to the protein content of the medium; (*b*) reaction to postosmication related to the protein content of the medium; and (*c*) ultrastructural morphology and chemical characteristics related to growth in suspension or as a sheet, but independent of the protein content of the medium.

The strain L cells served as controls in these experiments and did not vary appreciably in their ultrastructure from one medium to another.

Morphology Related to Protein Content of Medium

Certain mitochondria, nuclei, the Golgi material, and annulate lamellae show structural similarities in cells grown in both protein-containing and protein-free medium. These structures can now be considered in detail in the human skin cells.

The mitochondria show considerable variation in orientation of cristae and shape. Figures 1, 15, 16, 17, 24, and 25 demonstrate the typical forms assumed. The usual morphology of the mitochondria consists of an elongate double-membrane body with the cristae projecting transversely to the long axis. Less frequently the cristae are arranged parallel to the long axis (figs. 1 and 17). Although the entire range of variation is represented in both media, certain mitochondria are highly specialized in

structure in the cells grown on protein medium, and these will be described later.

The nuclei in both types of media have the usual double membrane and a granular structure. The only remarkable feature is the occasional presence of a small structure, limited by a single membrane, apparently within the nucleus (fig. 23). These structures assume various forms and in some instances have the appearance of mitochondria (*11*). Since there is only a single membrane surrounding them, they are assumed to be intranuclear or at least delineated by the inner nuclear membrane. They occur in cells grown in all the media used; thus no differential significance can be attached to them.

Figures 17 and 18 demonstrate the similarity in structure of the Golgi material in cells grown on both the protein and protein-free media. It should be noted that the Golgi apparatus is well developed in all the epithelial cells observed in this study.

Structures which appear identical to annulate lamellae [figs. 20 and 22 (*12*, *13*)] occur in cells grown in each medium. They are seen with equal frequency in all the media, though they are not numerous in any.

Certain morphological differences occur between cells grown in the two media. These differences are: 1) in the shape of the cells and in their growth pattern; 2) certain mitochondrial derivatives present in the protein-medium cells which are not present in protein-free medium; 3) a greater number of lipide vacuoles in protein than in protein-free cells; and 4) the presence of tonofibrils in cells grown in protein medium.

As has been stated earlier, when epithelial cells are grown in protein medium in glass flasks, they grow as a sheet of flattened cells, each in contact with its neighbor, and not more than 1 cell layer thick. The epithelial cells, when cultured in the protein-free medium, grow in suspension in this particular study, either singly or in small clumps of not more than 5 or 6 cells; all these cells are rounded rather than flattened.

Certain structures which shall be called *lamellar bodies* are noted in the cells growing in protein-containing medium, but never observed in protein-free medium (figs. 3, 4, and 6 through 15). In many instances the configuration of electron-dense layers is quite similar to that seen in myelin of peripheral nerve (*14*). In addition to the fully developed lamellar bodies, as shown in figure 4, there are all stages of complexity, which grade down to fairly simple structures arising from mitochondria (figs. 12, 13, and 14). The thickness of all these membranes is of the order of 80 to 100 A. Tonofibrils are seen only in cells grown in protein medium. Although they are not found as frequently in tissue-culture cells as in epidermal cells of intact skin, they are of the same dimensions and appearance (*15*). An occasional tonofibril is found in contiguous relationship with a lamellar body, since the latter appears to be a mitochondrial derivative (fig. 15). Lipide droplets are seen much more frequently in cells grown in protein than in protein-free medium (figs. 3, 4, 5, and 20). There is nothing remarkable in the appearance of the lipide when it does occur.

Postosmication Reactivity Related to Protein Content of Medium

When the postosmication treatment is carried out, both the Golgi complex and mitochondria of cells, which were grown in protein as well as protein-free medium, manifest some distinctive and probably significant differences from normal. The Golgi material does not reduce osmium tetroxide in the usual fashion (*10*) in cells grown in either type of medium (figs. 17 and 18). Although mitochondria do not normally reduce osmium tetroxide when this procedure is carried out, they do so with almost complete consistency when cells are grown in suspension in a protein-free medium (figs. 18, 19, 22, and 23), but not when the cells are grown in protein medium or in a sheet in protein-free medium (fig. 24). In the mitochondria which react, it can be seen that the reduction products of osmic acid are deposited between the outer and inner mitochondrial membranes and between the membranes making up the cristae (fig. 19). In some cells and in a few areas of an occasional cell grown on protein-free medium, and especially in young cultures, the mitochondria have not completely reacted to the postosmication procedure (figs. 2 and 23). Lamellar bodies are occasionally observed to reduce osmic acid, as are occasional unidentified structures of random form.

Certain structures other than mitochondria are occasionally post-osmicated in cells grown in either type of medium. Lamellar bodies are rarely seen to have taken up osmium as well as occasional unidentified structures of random form (figs. 15 and 21).

Morphology and Chemical Characteristics Related to Growth in Suspension or as a Sheet

The possibility was considered that the differences between cells grown on protein compared to protein-free medium were due to the surface form of the cells, *i.e.*, rounded in the protein-free and flattened in the protein medium. To determine if this consideration were correct, experiments were set up to grow the cells in a flat sheet in the protein-free medium with a Millipore filter on which cells could anchor themselves. The glass was coated with silicon in an attempt to force cells in the protein medium to grow in suspension, but this was not successful.

When a Millipore filter is present in the protein-free medium, the cells grow in sheets on it and assume a form similar to that of cells grown in the protein medium. When such cells are postosmicated, the mitochondria and Golgi bodies do not reduce osmium tetroxide. In general, the arrangement of elements in the cytoplasm is also similar to that of cells grown in protein medium (figs. 24 and 25). Therefore, it may be considered that a correlation exists between the surface form assumed by these cells and the chemical reactivity of the mitochondria to post-osmication.

DISCUSSION

This discussion will be concerned with: 1) the possible derivation of the lamellar bodies, 2) the significance of the highly developed Golgi apparatus, 3) a provisional interpretation of the altered postosmication reaction that occurs when cell morphology is changed, and 4) some minor observations.

A series beginning with typical-appearing mitochondria and passing through all stages of complexity to the fully developed lamellar body can be collected from cells grown in protein medium. The earliest stage in which it is apparent that a double-membrane terminal enlargement is developing from a mitochondrion is seen in figures 12 and 14. Figure 9 shows a somewhat more complex form in which it appears that most of the mitochondrion has developed into a vacuolated body, limited by two sets of double membranes. Figures 13, 6, 7, and 8 demonstrate in series the progressive addition of membranes, while figures 3, 4, 10, and 15 illustrate the great complexity which is ultimately achieved by the lamellar bodies.

It has been previously suggested that mitochondria may have an active role in keratin production (*15*). In view of the present observations that mitochondria may develop into lamellar bodies, the close relationship of tonofilaments to lamellar bodies (fig. 15) assumes significance. If keratin polymerized into the form of tonofilaments by some direct effect of mitochondria so that an actual attachment between the two is present, then this attachment could understandably persist when the involved mitochondria develop into lamellar bodies.

Dalton and Felix (*16*) have suggested several possible functions of the Golgi complex. One of them is that of intracellular osmoregulation. Previous observations have shown that the Golgi apparatus in the *in situ* epidermal cell is vestigial (*15*). It is obvious that the epidermal cell growing in a water-rich tissue-culture medium would be required to handle much more water than the same cell growing in the relatively dry environment of the epidermis. This could explain why the Golgi apparatus of the tissue-culture cell is highly developed.

The basis of the postosmication reaction is not completely known, though some information about its nature is available. The substance responsible for reduction of osmium tetroxide is not known, but a reasonable hypothesis for the high concentration of lipide in the Golgi zone would be that an unsaturated lipide is responsible. Such a lipide would probably be derived from a lipoprotein complex (*16*). This reaction is abnormal when it occurs in mitochondria. It may be that the lipoprotein complex of the normal mitochondrial membranes is so structured that the postosmication treatment does not disrupt the lipide-protein bonds, and thus no reactive groups of the lipide moiety become exposed. When conditions are changed in the environment of the cell so that the mitochondrial membranes assume a configuration such that the lipide-protein bonds are more easily broken, the characteristic postosmication reaction may occur (figs. 18, 19, 22, and 23). When cells are grown in protein-free medium in a rounded form, the mitochondria can be postosmicated, but when cells

are grown in the same medium in a flat form on a chemically inert filter, no such change occurs. It would seem that a mechanical change in surface configuration of the cell has resulted in a chemical change in the mitochondria. A similar change may occur in the mitochondrial membranes as the lamellar bodies are formed (fig. 15). If this hypothesis is correct, its significance in embryological studies is apparent.

The reason for the presence of reduced osmium in relation to mitochondria, and its absence in relation to the Golgi apparatus, is not clear. The demonstration of the Golgi complex in tissue-cultured cells by impregnation methods has been notoriously difficult, but it may well be related to the particular conditions of tissue culture used in the particular study (*17*).

Occasional unidentified structures take up osmium in the postosmication treatment, but they are rare and no information is available about them.

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PLATES

567420—60——10

All electron micrographs are of human skin epithelial cells.

PLATE 168

FIGURE 1.—Grown in chemically defined medium. Mitochondria appear typical. Dalton's fixative. × 37,200

FIGURE 2.—Grown in chemically defined medium, Palade's fixative, and post-osmicated. This represents the usual form of cells grown in the medium; when cells are not completely adapted to rapid growth in the chemically defined medium, they tend to be rounded up, and several vacuoles are present. × 7,180

FIGURE 3.—Grown in protein-containing medium, Palade's fixative, and post-osmicated. This is representative of cells grown in this medium insofar as they have close relationship to other cells, have lamellar bodies, and do not have the vacuolar cytoplasm of cells grown in chemically defined medium. × 6,800

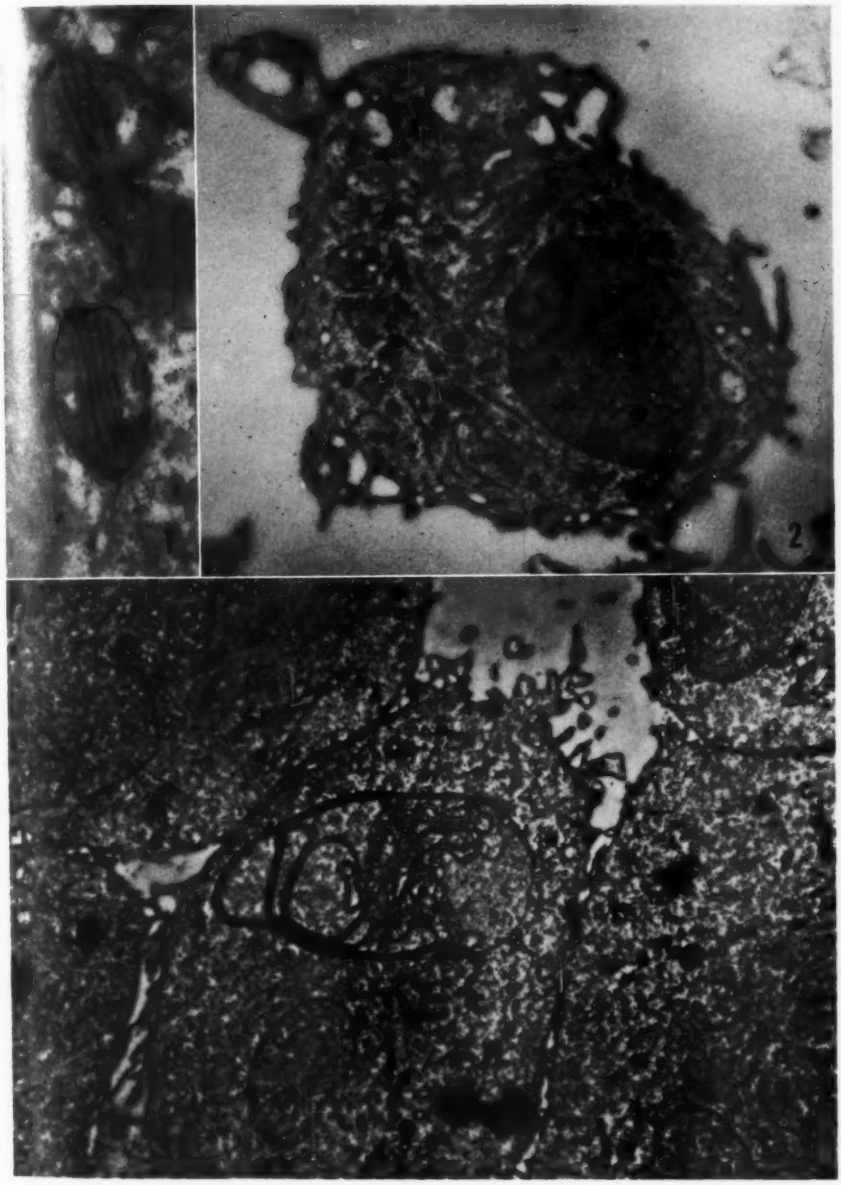


PLATE 169

FIGURE 4.—Grown in protein-containing medium, Dalton's fixative. Shows complex relations of membrane in a lamellar body. × 28,400

FIGURE 5.—Grown in protein-containing medium, Dalton's fixative. Shows cytoplasmic detail with mitochondria, lipide droplets, ergastoplasmic granules, and vacuoles of different kinds. × 38,200

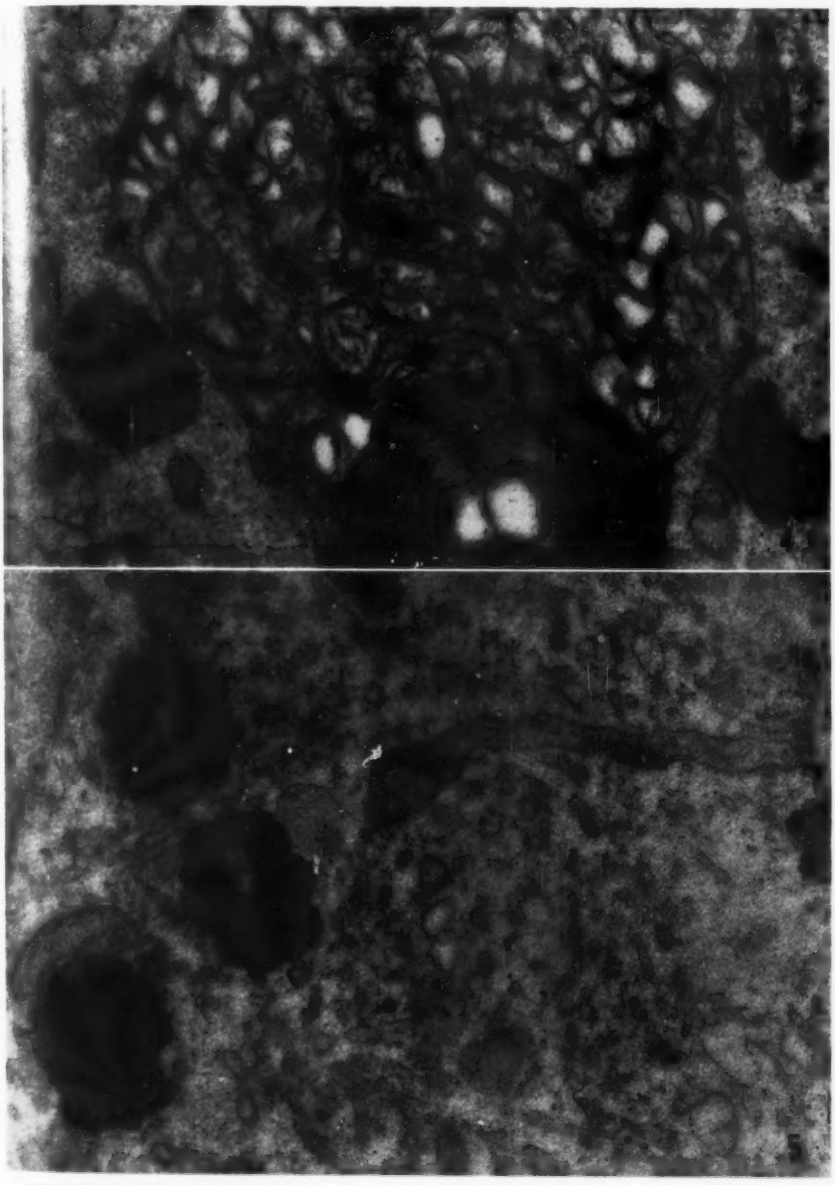


PLATE 170

FIGURES 6 through 14.—Grown in protein-containing medium. Structures in human epithelial cells show a series of stages from normal appearing mitochondria to fully developed lamellar bodies. Figures 12, 13, and 14 show structures which are like mitochondria on one end and which have multilayered enlargements on the other. Figure 9 shows a structure having some resemblance to a mitochondrion, but appearing more like a lamellar body. Figures 6 and 8 are typically small bodies. Figure 10 is a characteristic fully developed lamellar body. Figure 11 shows an interesting relation between a small lamellar body and Golgi body. Magnifications by figure number are: (6) × 38,200; (7) × 43,500; (8) × 33,400; (9) × 33,400; (10) × 15,750; (11–14) × 38,200

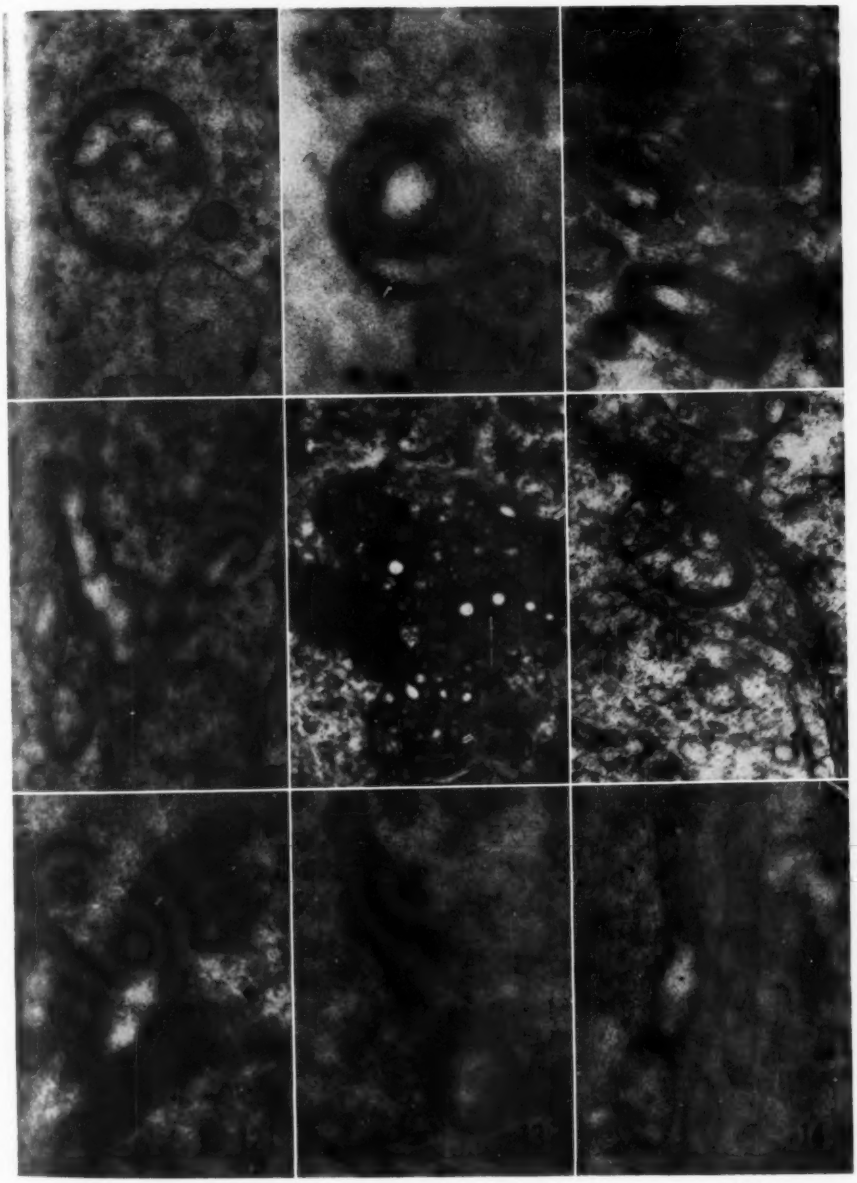


PLATE 171

FIGURE 15.—Grown in protein-containing medium, Palade's fixative, and post-osmicated. A lamellar body has taken up osmium. Tonofibrils can be seen in several places and are in close relation to lamellar body at one region (*arrow*). × 38,200

FIGURE 16.—Grown in protein-containing medium, Dalton's fixative. Demonstrates mitochondria, ergastoplasm, and tonofibrils. × 38,200

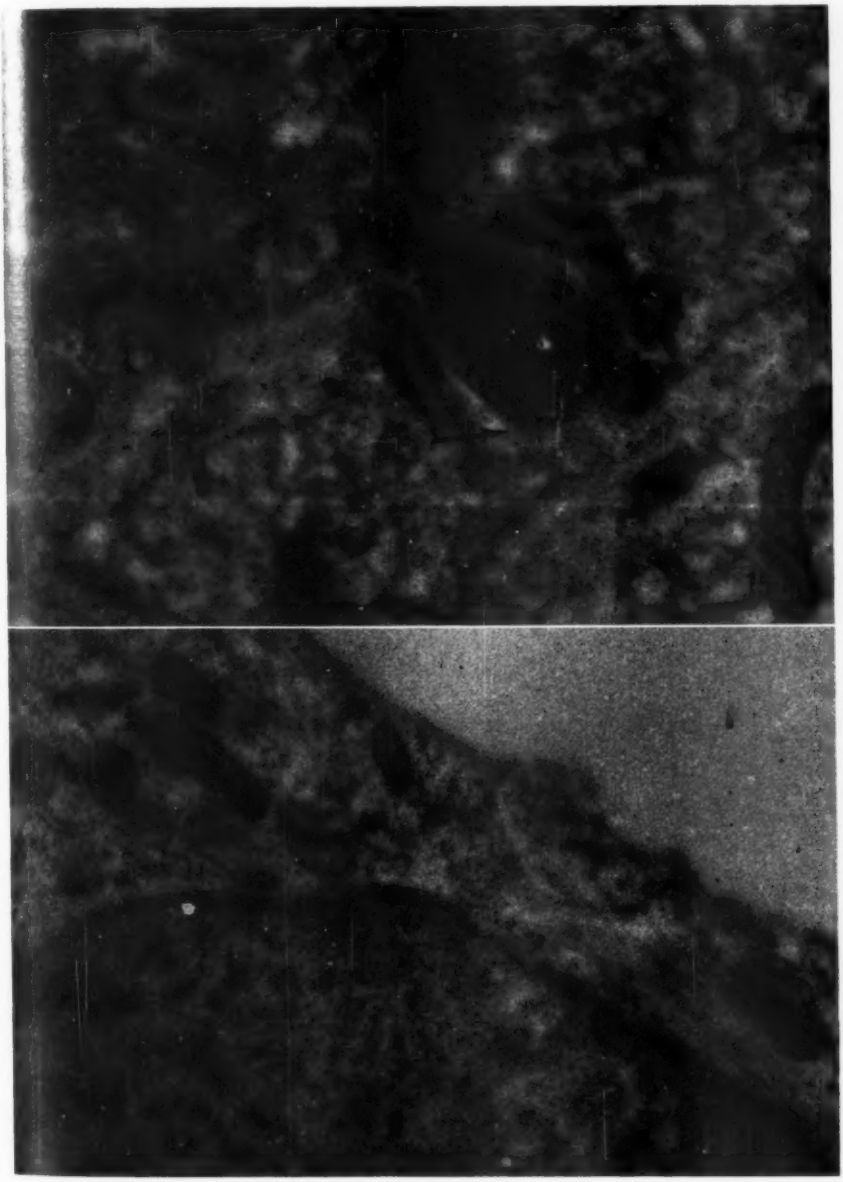


PLATE 172

FIGURE 17.—Grown in protein-containing medium, Palade's fixative, and post-osmicated. Golgi body and mitochondrion both appear normal. × 27,700

FIGURE 18.—Grown in chemically defined medium, Palade's fixative, and post-osmicated. Golgi body appears as usual, but osmium has been deposited between double membranes of several of the mitochondria. × 25,000

FIGURE 19.—Grown in chemically defined medium, Palade's fixative, and post-osmicated. Typical appearance of mitochondria which have reacted in the post-osmication procedure. × 29,600

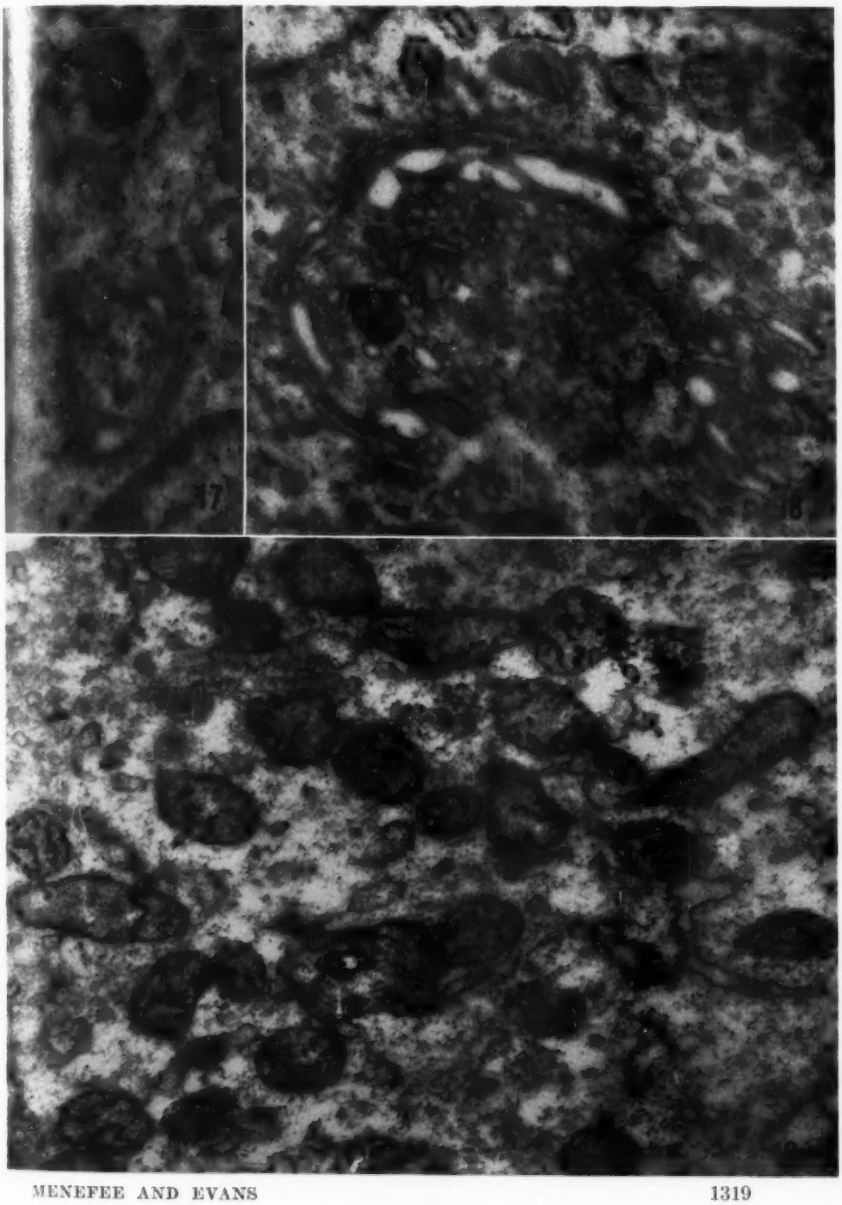


PLATE 173

FIGURE 20.—Grown in protein-containing medium, Dalton's fixative. Annulate lamellae of Rebuhn are seen here. Parts of nucleus are seen in *lower* and *left* parts of micrograph. × 30,600

FIGURE 21.—Grown in protein-containing medium, Palade's fixative, and post-osmicated. Parts of 2 cells separated by cell membranes can be seen. Golgi body and mitochondria in cell on *left* have not reacted, though an unidentified structure in cell on *right* has taken up osmium. × 18,150

FIGURE 22.—Grown in chemically defined medium, Palade's fixative, and post-osmicated. Mitochondria have taken up osmium, but the annulate lamellae have not. × 36,300

FIGURE 23.—Grown in chemically defined medium, Palade's fixative, and post-osmicated. This is a region in which mitochondria are beginning to manifest the chemical change which results in osmium deposition. × 30,550

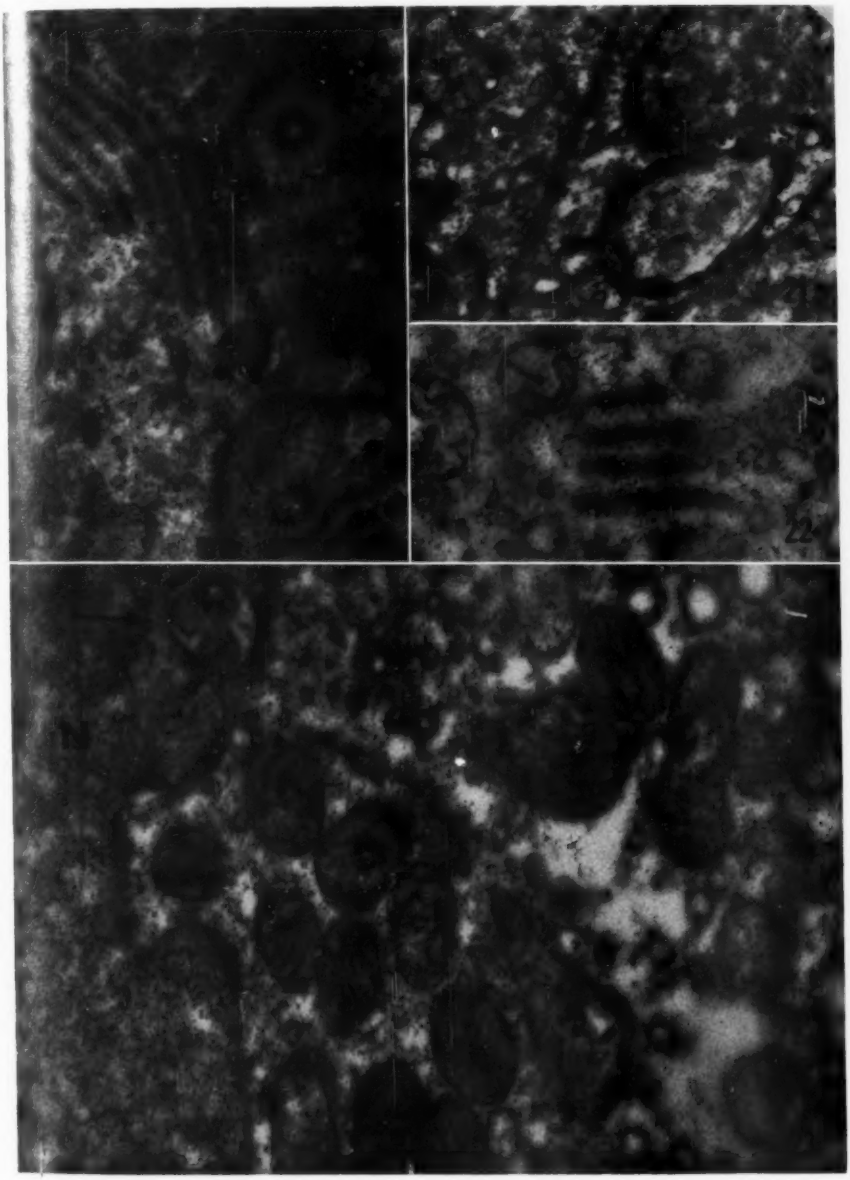
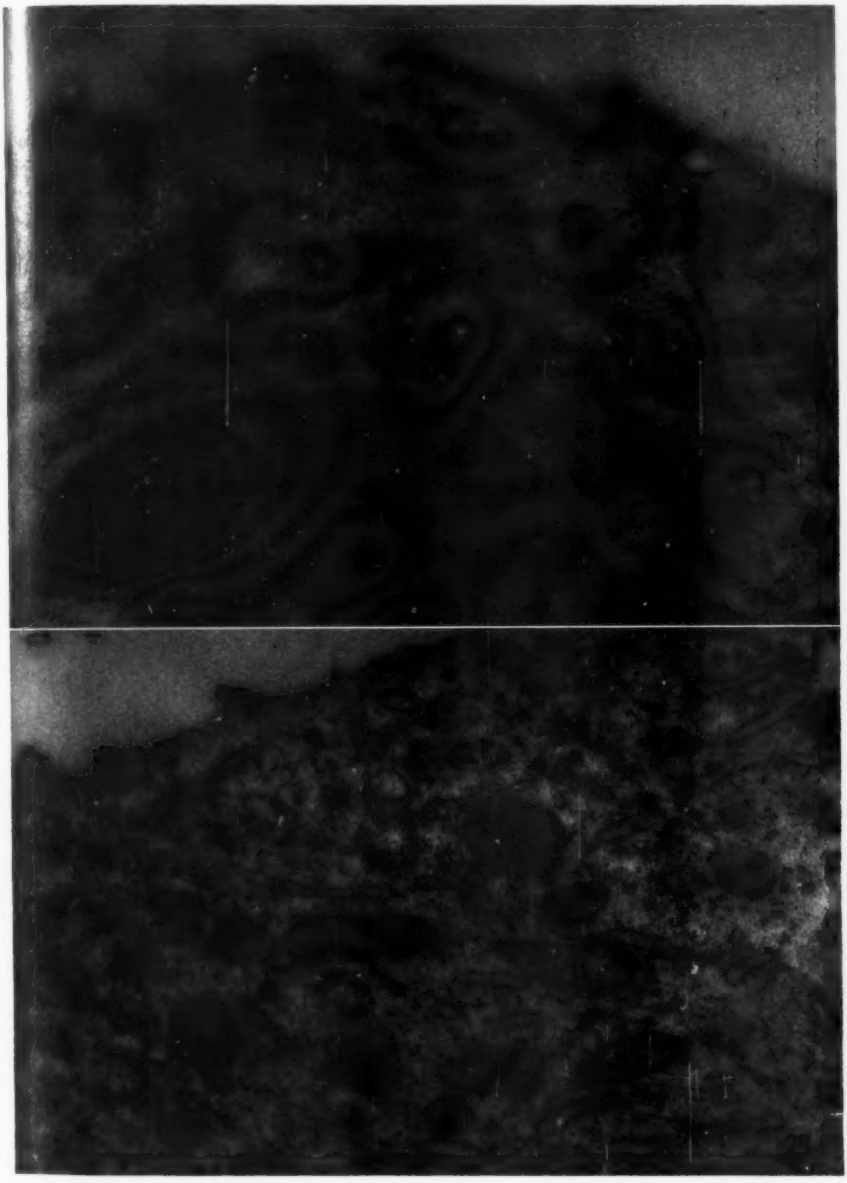


PLATE 174

FIGURE 24.—Grown in chemically defined medium, on Millipore filter, so that the cells grew in sheets rather than in suspension; Palade's fixative, postosmicated. *Note* that mitochondria do not take up osmium and that cytoplasm resembles that of cells grown on protein-containing medium rather than other cells grown in chemically defined medium without a filter. Nucleus (N) containing an inclusion at *arrow*. × 32,000

FIGURE 25.—Grown in protein-containing medium, Dalton's fixative. *Note* similarity between structures shown here and those in figure 24. A pentalaminar body can be seen at *arrow* (*18*). × 32,000.



Metabolism of Mammary-Gland Slices From Rats Receiving Progesterone and 3-Methylcholanthrene [1, 2]

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SUMMARY

Administration of 3-methylcholanthrene orally for as long as 62 days to young female albino rats receiving progesterone had no demonstrable effect, even in the presence of induced cancers elsewhere in the breast, on the values for the following metabolic parameters of mammary-gland slices: respiration, aerobic glycolysis, anaerobic glycolysis, and catalase activity, as compared to values obtained from control animals receiving either no treatment or sesame oil alone. Progesterone by itself, however, induced proliferation of mammary epithelium and the accompanying increase in Q_{O_2} was due to this proliferation, but after a peak was reached, the epithelium and respiration decreased to the level of controls. When 3-methylcholanthrene was administered with progesterone, the Q_{O_2} did not increase because the epithelium failed to proliferate. Liver slices from animals treated with progesterone alone had an increased and labile Q_{O_2}, an effect which was not observed in slices from animals receiving 3-methylcholanthrene in addition to progesterone. Livers from animals receiving 3-methylcholanthrene had an increased lipide content. Treatment of the host did not influence the respiration of kidney slices. Preputial-gland slices showed an extreme variation in Q_{O_2} unrelated to treatment. The earliest, and smallest, cancer nodules permitting study had the characteristic neoplastic type of metabolism.—J. Nat. Cancer Inst. 25: 1325–1337, 1960.

BY ORAL administration of an optimum dose of 3-methylcholanthrene, Huggins, Briziarelli, and Sutton (*1*) were invariably able to induce mammary tumors in female albino rats within 30 to 60 days. Many of the tumors were hormone-dependent, and progesterone shortened the induction period. Tumors induced in this manner have a high anaerobic and aerobic glycolysis (*2*), which Warburg showed was characteristic of the metabolism of neoplasms (*3*). The present study was undertaken to determine 1) whether there is a general or localized, abrupt or gradual change toward a neoplastic type of metabolism in the mammary gland

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during the administration of 3-methylcholanthrene, and 2) whether a sequence of metabolic events preceding overt neoplasia, such as that described by Warburg *et al.* for tissue-culture cells (*4*), could be observed.

MATERIALS AND METHODS

Female Sprague-Dawley rats, with an average weight of 150 g, were obtained at 44 days of age. After resting for 11 days, the animals were numbered and divided into 4 groups by use of a table of random numbers. The following regimens were then instituted: The animals in each group received food pellets and water *ad libitum*, and fresh orange daily. The 5 rats in the untreated control group received nothing else. The sesame oil control group consisted of 4 rats that were given 1 ml of sesame oil 6 days a week through a gastric tube. The progesterone group consisted of 7 rats that received a daily intramuscular injection of 0.2 ml of sesame oil containing 4 mg of progesterone, 6 times a week. The 3-MC-progesterone [4] group of 7 rats, in addition to the intramuscular injection of progesterone, received 10 mg of 3-MC in 1 ml of sesame oil by gastric tube, daily, 6 times a week. During the subsequent 7 weeks, animals were decapitated and metabolic determinations made on slices prepared from mammary gland, liver, kidney, preputial gland, and tumor (when present). Animals are designated by the group and treatment day on which they were studied. Treatment was never given on the day of an experiment, to avoid possible acute effects, and the animals were not fasted.

Slices were prepared by the method of Deutsch (*5*). Respiration was measured manometrically at 37° C in Warburg flasks containing 0.2 ml of 20 percent KOH in the center well, with O_2 as the gas phase. Ringer's phosphate solution, at *p*H 7.4, containing 0.2 percent (w/v) D-glucose but no calcium, served as the incubation medium (*5*). Anaerobic glycolysis was determined manometrically in Ringer's bicarbonate medium with 95 percent N_2–5 percent CO_2 as gas phase. Aerobic glycolysis was estimated by the direct method (*6*) with the use of Ringer's bicarbonate medium and 95 percent O_2–5 percent CO_2 and assuming an RQ of 1.0. The Ringer's bicarbonate medium did not contain calcium, but did contain 0.2 percent (w/v) D-glucose. Upon termination of the experiment, the slices were washed in distilled water, blotted, and then dried overnight in a 105° C oven. Fat was extracted from the dry tissue by refluxing with carbon tetrachloride overnight. Q values were calculated as microliters per milligram of dry nonfat tissue per hour. Mean values are accompanied by ± their standard error, unless specified otherwise. The mean Q_{O_2} values cited are those obtained during the first hour of measured respiration, but values for aerobic glycolysis, $Q_A^{O_2}$, and anaerobic glycolysis, $Q_A^{N_2}$, are those obtained during the first ½ hour of measurement.

[4] Abbreviations: 3-methylcholanthrene (3-MC); milligram tissue nitrogen (mg N); dry nonfat tissue (dnft); and mg of dnft/mg N (dnft/mg N).

Nitrogen determinations were made by the microKjeldahl method after digestion in a copper sulfate-selenous acid mixture. The protein content of the medium at the end of the incubation period was estimated by the Lowry-Folin method (*7*), with bovine serum albumin as a standard. The hydroxyproline content of the slices was determined, after hydrolysis in 6 N HCl in a sealed tube for 14 hours at 105° C, by the method of Neuman and Logan (*8*). An estimate of catalase activity was obtained manometrically by the method used by Warburg (*9*) for tissue-culture cells. At the end of the 2-hour period of respiration measurement, 0.1 ml of H_2O_2 in a concentration sufficient to give a final concentration of 0.04 M was dumped into the incubation medium from the sidearm. The flasks were shaken at 120 cycles/minute. Readings were made at 10-second intervals, and the initial slope (μl/minute) was determined after the points had been plotted on graph paper. At least 95 percent of the catalase activity measured was associated with the protein lost to the medium rather than with the intracellular proteins of the intact slices; this was expected since diffusion into the slice and cells is a limiting factor. Consequently, the catalase activity was calculated as μl O_2 evolved per minute by 1 mg of medium protein, as determined by the Lowry-Folin method. This procedure was convenient and provided values of catalase activity comparable to those obtained from homogenates of the same tissue. Due to the complication of diffusion across a gas-liquid interface, catalase-activity determinations by the manometric method used are not suitable for some purposes (*10*); however, with standardized conditions and rapid shaking (120 cycles/minute), the precision obtained was adequate and the method satisfied the needs of the study, *i.e.*, the ability to demonstrate any gross changes or relative differences in catalase activity.

RESULTS

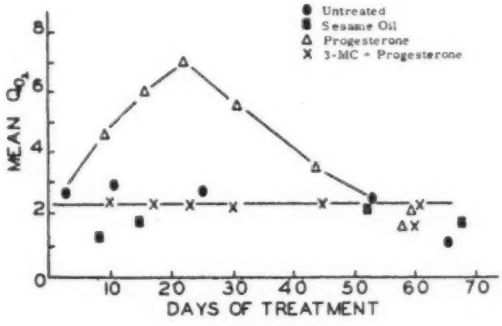
General

On the 4th day of treatment the coats of the 3-MC-progesterone group appeared coarse and much less sleek, and the animals did not appear as active as the controls. From the 19th to 21st days of treatment, there was remarkable improvement in the appearance of their coats and the animals seemed more active. Subsequently, the coats and the activity of the animals in this group were not noticeably different from the untreated controls. The progesterone group maintained exceptionally fine coats and gained more weight than did animals in the other groups. With respect to the gross appearances of the organs, the only unusual observations were the following: (*a*) In the 3-MC-progesterone group the livers of the animals were tan, reminiscent of fatty livers. (*b*) The progesterone-group animals killed on the 22d and the 31st days of treatment had large, yellow-tan mammary glands which contrasted sharply with the thinner, white glands found in the other animals. In addition,

the animal treated with progesterone for 22 days had an enlarged preputial gland filled with a large amount of dark-olive, waxy substance, as did the animal of the same group treated for 44 days. The preputial glands of these 2 rats contrasted with the smaller, light-tan glands containing only a small amount of milky, thin material found in all the other rats. (*c*) The animal treated with progesterone for 58 days had a unilateral hydrosalpinx and a rectal polyp.

Mammary Gland

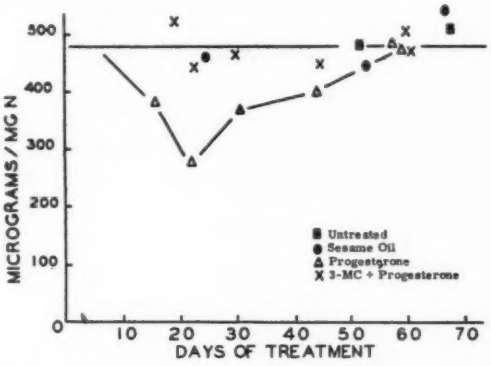
The fat content of the mammary gland was quite high in all the animals; the values ranged from 80 to 92 percent of the dry weight. No difference in the fat content of mammary glands from the control, sesame, and progesterone groups was noted, but during the first 45 days of treatment the fat content of glands from the 3-MC-progesterone group was 4 to 10 percent greater than that of the other groups. Q_{O_2} data for the rats from the untreated, sesame, and 3-MC-progesterone groups formed a



TEXT-FIGURE 1.—Respiration of mammary-gland slices.

baseline with a mean of 2.1 ± 0.1 (text-fig. 1). The Q_{O_2} of slices from the progesterone group, however, increased to a value of 7.0 in the rat treated for 22 days and then progressively decreased to the baseline value of rats treated for 52 days, a response to progesterone not observed when 3-MC was administered concomitantly. The aerobic glycolysis was determined on slices from rats treated for 44 to 67 days. The mean value was 1.1 ± 0.1 and no difference due to treatment was noted. For anaerobic glycolysis the mean value was 4.6 ± 0.3, and no difference due to treatment was noted. The hydroxyproline content of the mammary-gland slices is presented in text-figure 2. A baseline was formed by the values for the untreated, sesame, and 3-MC-progesterone groups, whereas the values for the progesterone group, before the 58th-day animal, were below the baseline and reached a minimum in the 22d-day animal. These results were the inverse of those from respiration studies and the signifi-

cance of this will be discussed later. The dnft/mg N ratio was 7.2 ± 0.2 in 61 determinations on separate mammary-gland slices. No effect of treatment on this ratio was evident. Protein lost from the mammary-gland slices of the progesterone group increased to a maximum of 13 percent of the total dry nonfat tissue for the rat treated for 22 days, and then gradually returned to the baseline value of 5 to 8 percent formed by the values from rats in the other groups. Anaerobic conditions did not significantly affect the amount of protein lost. The mean catalase activity of mammary gland was 366 ± 26.



TEXT-FIGURE 2.—Hydroxyproline content of mammary glands.

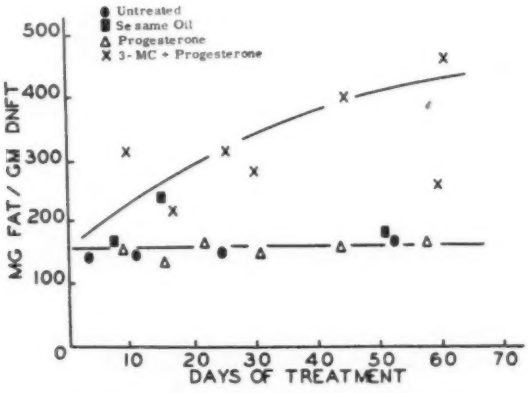
Tumor

Table 1 summarizes the data on respiration and glycolysis obtained from tumors which developed in the mammary glands of rats on the 3-MC-progesterone regimen. The first tumor was detected on the 45th day of treatment and was studied 5 days later. This solitary nodule was removed along with some of the adjacent mammary gland and sliced, but the amount of tissue was small. From the observed fat content and Q_{O_2} of the mammary gland remote from the tumor, and from the observed fat content and Q_{O_2} of the tumor-mammary gland mixture, it was possible by means of an algebraic formula to estimate the proportion of tumor tissue in the mixture and thus the Q_{O_2} of the tumor portion, if one assumes that the fat content of the dry tumor is the same as that in larger tumors: 10 ± 3 percent. The uncertainty intervals associated with the calculated results were approximated from the mean deviation associated with the experimental values used in the calculations. In the two later experiments (60th and 61st days), the tumors were multiple, larger, and had been detectable by palpation longer at the time of the study (13 and 14 days, respectively). Q_{O_2} measurements in these experiments were made on two different types of tissue mixtures; one flask contained tissue which was almost entirely tumor and the other contained tissue composed mostly of mammary gland adjacent to the tumor and only a small amount of tumor tissue. In each case the Q_{O_2} of the tumor portion was estimated by

the calculation outlined. Estimates for aerobic and anaerobic glycolysis were similarly obtained (table 1). Catalase activity for the predominantly tumor tissue was 150 ± 10, while the mean activity of mammary tissue remote from the tumor was 351 ± 56. This decrease of over one half was significant ($P < 0.01$), and since the value was not corrected for the "contamination" of the tumor by mammary gland, it connotes a higher activity for the tumor than it really possessed. The mean catalase activity of tumor tissue was 109 ± 12 when large tumors not contaminated with mammary gland were studied.

Liver

In accord with their appearance, the livers of animals from the 3-MC-progesterone group had an increased amount of fat; though variable, the fat content seemed to increase progressively but not linearly with time (text-fig. 3). Since Kjeldahl nitrogen values were obtained on all liver



TEXT-FIGURE 3.—Content of lipide in liver.

slices, the data in text-figure 3 were corrected to the dnft/mg N values obtained from fasted animals: 7.3 ± 0.1. The presence of hepatic glycogen would increase the dnft/mg N ratio, and thus these ratios permit an estimate of the liver glycogen content of the nonfasted animals. The slices used in the respiration studies almost invariably had a value in the range of the fasted livers, whereas pieces of the same liver that were not incubated but rather were maintained stationary at 4° C during the experiment had values up to 8.7, which indicates in this case a glycogen content of over 200 mg/g of dry nonfat liver. Estimated in this way and without an effort to maintain the glycogen in the liver at the time of death of the animal, the values were quite variable. However, the following statements can be made: (1) The maximum estimated glycogen values were in the normal range of 200 mg percent or over; (2) high values were noted in animals on all 4 regimens so that there is no evidence for any

TABLE 1.—Metabolism of early cancer nodule

Days on 3-MC	Age of detectable tumor (*days*)	Observed values: Mammary gland remote from cancer nodule (*a*) — Fat in dry tissue (*percent*)	Observed values: Mammary gland remote from cancer nodule (*a*) — Q determination ($\mu l/mg/hr$)	Observed values: Tumor + adjacent mammary gland (*b*) — Fat in dry tissue (*percent*)	Observed values: Tumor + adjacent mammary gland (*b*) — Q determination ($\mu l/mg/hr$)	Values derived by calculation: Tumor alone (*c*) — dnft Studied (*percent*)	Values derived by calculation: Tumor alone (*c*) — Q values ($\mu l/mg/hr$)
45	5*	91.5 ± 1.5	$Q_{O_2} = 2.6 \pm 0.1$	82.8	$Q_{O_2} = 4.6$	56	$Q_{O_2} = 6.0 \pm 2$
61	13†	89.9 ± 3.4	$Q_{O_2} = 1.6 \pm 0.1$	84.4	$Q_{O_2} = 3.4 \pm 0.3$	40	$Q_{O_2} = 6.2 \pm 5$
			$Q_A^{O_2} = 1.2 \pm 0.1$	78.0	$Q_{O_2} = 8.3 \pm 0.4$	61	$Q_{O_2} = 12.6 \pm 2$
			$Q_A^{N_2} = 4.8 \pm 0.1$	59.4	$Q_A^{O_2} = 4.4 \pm 0.1$	85	$Q_A^{O_2} = 5.0 \pm 1.0$
				56.7	$Q_A^{N_2} = 21.7 \pm 0.7$	86	$Q_A^{N_2} = 24.3 \pm 5.5$
62	14†	89.7 ± 1.4	$Q_{O_2} = 2.2 \pm 0.1$	84.6	$Q_{O_2} = 5.2 \pm 0.1$	39	$Q_{O_2} = 9.9 \pm 3$
			$Q_A^{O_2} = 1.1 \pm 0.4$	39.9	$Q_{O_2} = 9.8 \pm 0.1$	94	$Q_{O_2} = 10.4 \pm 1.5$
			$Q_A^{N_2} = 2.4 \pm 0.1$	43.9	$Q_A^{O_2} = 6.4 \pm 0.3$	92	$Q_A^{O_2} = 6.9 \pm 1.2$
				55.2	$Q_A^{N_2} = 16.4 \pm 1.4$	87	$Q_A^{N_2} = 18.5 \pm 4$

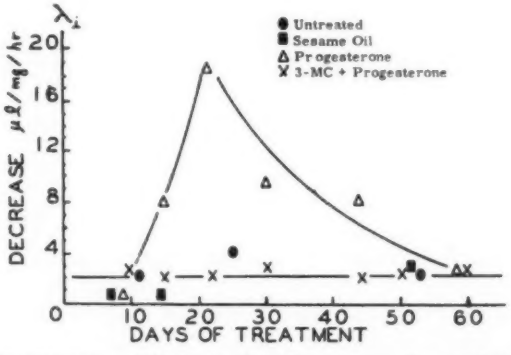
*Single tumor.
†Multiple tumors.

Manometric determinations of respiration (Q_{O_2}), aerobic glycolysis ($Q_A^{O_2}$), and anerobic glycolysis ($Q_A^{N_2}$) were performed on slices from (*a*) mammary gland remote from a cancer nodule, and (*b*) a mixture of the early cancer nodule plus adjacent mammary gland. Q values are expressed in μl/hour/mg of dry nonfat tissue, designated dnft. From the observed percent fat values for (*a*) and (*b*) and the mean fat content of dry tumor, *i.e.*, 10 ± 3 percent, the content of and the Q values for the tumor component in (*b*) were calculated. Mean values ± mean deviations are presented in (*a*) and (*b*), whereas in (*c*) the calculated values are presented. The uncertainty intervals in (*c*) were derived from the mean deviations associated with the observed values used in the calculations. Each observed value represents 1 to 4 determinations.

failure in hepatic glycogen deposition due to treatment; and (3) the low values for the incubated slices in all groups indicated no gross impairment of glycogen utilization.

With the exception of animals in the progesterone group, the Q_{O_2} values of liver slices were remarkably uniform with a mean of 9.9 ± 0.3. Liver slices from animals in the progesterone group had a variable but significant ($P < 0.01$) elevation of Q_{O_2} with a mean of 12.8 ± 1.3. In addition to this increased mean Q_{O_2} value, the respiration of liver slices from progesterone-group animals had another unusual characteristic. Although the Q_{O_2} of slices from animals in other groups decreased gradually and slightly (0–1 μl/mg/hr) during the 2-hour incubation period (the rate of decline was slightly greater during the first 15–30 minutes than later), liver slices from progesterone-group animals frequently exhibited a marked initial drop in Q_{O_2} during the first 15 to 45 minutes of measurement before leveling off to a more gentle decline similar to that observed in the other slices. Data illustrating this behavior are represented in text-figure 4. By plotting on graph paper the manometer reading versus time of the reading, measuring the slope directly and expressing it as Δ μl O_2/hour/mg of dry nonfat tissue, we obtained the slope (rate) of the initial decline, λ_i. On the same plot it was also possible to extrapolate the respiration to zero time, *i.e.*, the start of the measuring period, and thus obtain a value for the initial rate of respiration, designated $Q^{\circ}_{O_2}$. The mean value for the $Q^{\circ}_{O_2}$ of slices from animals in the progesterone group was 16.0 ± 1.6, whereas the mean value was 11.4 ± 0.3 for slices from animals in the other groups ($P < 0.01$).

The mean catalase activity of liver was 2260 ± 250 for the progesterone group and 2740 ± 170 in the other groups ($P = 0.06$).



TEXT-FIGURE 4.—Initial rate of decline of liver-slice respiration.

Kidney

The mean Q_{O_2} for kidney slices was 23.4 ± 0.6 and was constant for the 2-hour period of measurement. In contrast to mammary gland and liver, no effect of treatment was noted. Catalase activity was 1800 ± 160.

Preputial Glands

The respiration of slices from the preputial glands was extremely variable, with Q_{O_2} values ranging from 3 to 11. The variation seemed to be independent of treatment, including progesterone. Anaerobic glycolysis, however, was relatively uniform with a mean value of 3.8 ± 0.3, and, again, no effect of treatment was noted. Catalase activity was constant throughout the study at 223 ± 17, and was not influenced by treatment or by anaerobic incubation. The mean fat content was 37.8 ± 6.6 percent of the dry weight; and the dnft/mg N ratio of 6.5 ± 0.1 was quite low compared to the 7.3 ± 0.2 value of other tissues.

DISCUSSION

Mammary gland contains essentially three different tissue components: adipose, epithelial, and connective. Dry retroperitoneal adipose tissue had a fat content of 98.5 percent (or 1.5% nonfat) and a dnft/mg N ratio of 7.5. With the use of these values, the adipose-tissue respiration data of Jungas and Ball (*11*) and Haugaard and Marsh (*12*) were converted from a tissue-nitrogen and wet-tissue basis, respectively, to a Q_{O_2} value based on dry nonfat tissue, and a Q_{O_2} value of 12 ± 1 was obtained. If 80 percent of the dry weight of a mammary-gland slice is fat, then 0.06 mg, *i.e.*, $(0.015 \times 0.80)/(1.00-0.80)$, of each mg of dry nonfat tissue is from the adipose component. Similarly, if the fat content is 90 percent, the adipose contribution is 0.135 mg. The adipose component of mammary gland can thus contribute 0.7 μl/hour (*i.e.*, 12×0.06) to 1.6 μl/hour (*i.e.*, 12×0.135) to the observed Q_{O_2} of mammary glands having fat contents in the 80 to 90 percent range. Connective tissue has a Q_{O_2} value of about 0.7 (*13–15*). If mammary gland consisted only of adipose- and connective-tissue components, then the corresponding Q_{O_2} range would be 1.4 to 2.2. It is evident (text-fig. 1) that most of the basal values are in this range, and thus the adipose- and connective-tissue components can account for the bulk of the respiration observed in the virgin (nonproliferated) mammary gland.

Collagen contains hydroxyproline, an amino acid not found in appreciable amounts in other mammalian proteins, and thus the hydroxyproline concentration of a tissue provides an index of connective-tissue content. The proliferation of epithelial cells relative to connective tissue has the effect of decreasing the hydroxyproline concentration of the tissue. The respiration rate of mammary-gland slices varied inversely and linearly with hydroxyproline concentration—thus the rate increased directly with the proportion of the epithelial-cell component—and the mammary epithelial-cell component alone had a Q_{O_2} value of 14.2 ± 1.8 (*16*). The respiration rate intrinsic to the mammary epithelial-cell component, which presumably is the cell type of origin of the mammary adenocarci-

noma induced by 3-methylcholanthrene, is thus comparable to the respiration rate of mammary cancers from hosts unmodified endocrinologically (*2*). Such cancers have a mean Q_{O_2} of approximately 13.6 when corrected for the connective tissue present. However, despite the correspondence in respiration rates, the respiration of the mammary cancers frequently responded markedly to changes in the endocrine state of the host (*2*), whereas under the same conditions mammary epithelium generally did not.

The marked increase in Q_{O_2} of mammary-gland slices from animals in the progesterone group was accounted for by the relative increase in the epithelial component (*16*). 3-MC administered with the progesterone, however, prevented any measurable proliferation of epithelium and, therefore, the accompanying increase in respiration rate did not occur. There was no evidence that 3-MC inhibited the respiration of any of the individual tissue components of breast.

The small amount of epithelium and low respiration rate of virgin breast tissue from untreated animals present difficulties for the study of depressing influences on the epithelial components of such breasts. The following considerations illustrate this: If 4 percent of the dry nonfat tissue of virgin mammary gland (a reasonable estimate of the upper limit) were contributed by epithelium, then approximately 0.56 μl/hour would be contributed to the breast Q_{O_2} by epithelium; for 2 percent, 0.28 μl/hour; for 1 percent, 0.14 μl/hour, etc. (*16*). Since the baseline values (text-fig. 1) for the Q_{O_2} of mammary slices provide a mean of 2.1 with a standard deviation of $\pm$ 0.44 and a range of values from 0.8 to 3.0, it is evident that the epithelial content of mammary gland could be depressed many-fold without the accompanying change in respiration being experimentally demonstrable as significant. Since the bulk of the baseline respiration of breast is contributed by the connective- and adipose-tissue components, the contribution of the epithelial component is low, and thus even large relative decreases in epithelium produce only small absolute changes in respiration that are of the magnitude of the experimental variations. Analogous considerations apply to hydroxyproline determinations, since hydroxyproline was a marker for the bulk component (connective tissue) rather than the trace (epithelial) component. The advantage of progesterone administration was that proliferation occurred at levels where significant differences could be demonstrated between the situations in which 3-MC was and was not given; the disadvantage was the increased complexity of the experimental design and interpretation. The feature of decay of the proliferative response, during continuance of progesterone administration, was unexpected but of much interest, occurring as it did with other changes on about the 20th day of treatment when the rats were 75 days old.

In contrast to the Q_{O_2} results, anaerobic glycolysis and catalase activity were not uniquely correlated with the proportion of the epithelial component of mammary gland. The loss of protein to the incubation media varied directly with the epithelial proportion of mammary gland,

but this indicated only that the loss from epithelial tissue was greater than from connective tissue.

Like mammary-gland slices but unlike kidney slices, the respiration of liver slices was influenced by treatment of the host. Liver slices from animals in the progesterone group generally had an increased, though labile, respiration rate, but these characteristics were not observed when the animal received 3-MC as well as progesterone. Despite uniform rates of anaerobic glycolysis, the respiration rates of preputial-gland slices were remarkably variable, but no pattern was evident in this variability, nor was there any obvious relation to treatment or age of the rat. However, in view of (1) the profound influence of growth hormone and of androgenic steroids on the preputial glands of hypophysectomized rats (*17*), and (2) the influence of host-administered progesterone on the respiration rates of liver and mammary-gland slices, it is possible that the respiration rate of preputial gland may vary with the endocrine state of the host and may be correlated with the epithelial : connective-tissue ratio.

Although this 3-MC-induced mammary cancer has a much higher rate of aerobic and anaerobic glycolysis and a lower catalase activity than mammary gland, no changes were observed in these metabolic parameters of mammary glands during the 62 days of 3-MC administration, even in the mammary glands of hosts bearing the cancer. Within the sensitivity and precision of the methods used in this study, cancers in their earliest, and smallest, stage permitting study had the characteristic neoplastic type of metabolism; mammary glands, whether adjacent to or remote from the neoplasms, whether from hosts with or without cancers, whether from hosts receiving 3-MC at the time of study or not, retained their characteristic values for the metabolic parameters studied. Thus there was no indication that the mammary cancer induced by 3-MC occurred as a result of any gradual or generalized effect of 3-MC on the nonproliferating mammary gland. However, this histologically and biochemically well-differentiated adenocarcinoma is almost certainly of epithelial-cell origin, and the mammary glands in which the cancers arose had epithelial contents which were quite low, relative to the other tissue components, as judged by histological and biochemical criteria. Since the neoplastic process seems to arise in the acini and small ducts of the breast (*1*), the susceptible epithelial cells may be pictured, to a first approximation, as distributed throughout the virgin mammary gland in dispersed, small, discrete clusters of a relatively few cells. It is in such small regions that the initial carcinogenic event most likely occurs, as well as the events following it which lead on to an overt, gross neoplasm. Unless these changes (1) are sufficiently abundant throughout the mammary gland, (2) possess some unique property lending itself to sensitive assay, or (3) are associated with some generalized effect capable of study in the mammary gland, the changes will be diluted by the sheer bulk of the rest of the gland to the extent that the magnitude and abruptness or gradualness of changes cannot be definitely established. Of these condi-

tions facilitating study of the early events in carcinogenesis, it should be noted that with regard to the first (for this strain of rat on this dose schedule) only 3 to 6 tumors generally occur per animal; the second is a matter for further study, and the third receives no support from the results presented. Such considerations illustrate anew the difficulties involved in the use of a multicomponent tissue for the study of phenomena which may be confined to only one of the components. The ability to isolate or culture cells of the component of interest, or to measure some unique property of the component which can be correlated with the biological activity of interest in a manner analogous to that involved in action spectrums, can be of decisive importance.

3-MC is a carcinogen that is capable of causing sarcomas and carcinomas in various tissues, depending on the method and location of application. When administered orally, however, mammary adenocarcinomas greatly predominate, and this may reflect in part the chemical structure of 3-MC and the histological structure of mammary gland. 3-MC is a hydrocarbon, hence hydrophobic, and thus likely to be deposited and accumulated in body tissues containing a high fat content. Mammary gland obviously qualifies as such a tissue; the importance of mammary fat in this regard has also been suggested by Dao *et al.* (*18*), who devised a spectrofluorometric method for detecting tissue 3-MC. In mammary gland, 3-MC accumulation would surround the clusters of susceptible epithelial cells scattered throughout the extensive area of tissue. It is established that tumor incidence increases and tumor-appearance time decreases as the oral dose of 3-MC increases to the point of toxic and lethal effects (*1*); a simple interpretation of this finding is that the chance of a carcinogenic event occurring is related to the number of epithelial cells exposed and to the intensity and duration of exposure, *i.e.*, the integrated area under the 3-MC mammary concentration : time curve. Although this concept implies nothing about the nature of epithelial susceptibility, the number of events occurring for each tumor that appears, or what further requirements for survival and tumor development there may be, it does lend itself to quantitative study and formulation and, coupled with the observed proliferation effect of progesterone when given alone, permits a suggestion as to how progesterone shortens the mean time for tumor appearance, *i.e.*, by increasing the number of epithelial cells exposed and, in turn, the likelihood of a successful carcinogenic event occurring within a given interval of time.

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Mammalian Chromosomes *In Vitro*. XIII. Cyclic and Directional Changes of Population Structure [1,2]

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SUMMARY

Cells of strain L-P59 of the mouse multiply to at least twice as many cells as those of two L-P59 derivatives, strains Amy and Barbara. In mixed populations with an initially equal proportion of either L-P59 and Amy or L-P59 and Barbara cells, L-P59 cells completely dominated within 4 to 8 weeks. A marker chromosome, known as "D," was one of the most conspicuous elements in cells of L-P59. Cells contained 0, 1, 2, and 3 D chromosomes. In young cultures, mitotic cells with 0 or 1 D chromosome dominated the population, whereas in aged cultures those with 2 or 3 D chromosomes dominated it. When cultures of L-P59 were subcultured twice a week, the populations shifted to a lower D chromosome content (average D chromosome per cell below unity). Conversely, when cultures were subcultured once in 2 weeks without intervening feeding, the population shifted to a higher D chromosome content (average D chromosome per cell above 1.5). Also, when a young culture was suddenly treated with aged medium, the D chromosome content also increased among the mitotic cells. The results demonstrate that cell populations are composed of numerous genomes which are harmoniously balanced. Under one environmental condition, one or several genomes become the stem cells, but other genomes are able to replace them whenever conditions change.—J. Nat. Cancer Inst. 25: 1339–1353, 1960.

WITH THE firm establishment of chromosomal polymorphism (*1–3*) in cell populations of mammalian cell strains, investigators attempted to find correlation between certain physiological phenotypes and cytological features (*4–6*). However, such efforts proved fruitless. Koller (*7*) questioned the validity of the notion that these two phenomena must coincide. We agree with Koller's argument that there need not be any correlation between particular physiological characteristics and demonstrable chromosomal abnormalities. Not all the chromosomes are morphologically distinguishable in most cell populations, and the genes for the specific traits do not have to be located at marker chromosomes. Even

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if all chromosomes are recognizable, as in cells of Chinese hamsters, gene mutations do not show cytological abnormalities, unless the word "mutation" is used in the broad sense. Actually, investigators trying to find such correlation realized that such attempt would have little chance of success, but they designed the experiments hoping to get a "lucky break." Those who did obtain such a correlation (*8*, *9*) would have to regard the results a "fluke" (*10*). The avenue of attack, *viz.*, selecting a specific genetic behavior from a cell population and then checking for chromosomal specificity, is indeed unrewarding.

Theoretically, chromosomal material influences cell physiology. The D chromosome of strain L-P59 of the mouse was recorded to vary from 0 to 2 per cell (*11*). Let us assume that cells without the D element actually contained all the necessary genes but not in the form of the D chromosome, so that there was no loss of genes. Cells with two D chromosomes would, however, contain twice as many of these gene doses as those containing one D, while genes in other chromosomes remain the same. Unless the chromosome is completely inert, some physiological differences must exist between these two types of cells. The problem is how to demonstrate such differences. It would be a blind search if one attempted to assay all the known enzymes or other biochemical systems.

Some crude information can be obtained, however, if one does not specifically test a single physiological trait. Working on cell populations from a different viewpoint, and assuming that different genomes possess differential growth potentialities and adaptational abilities with the stem line being the most vigorous and best adapted to the cultural system employed, Hsu and Kellogg (*12*) treated the populations of strain L-P59 with colchicine to poison the stem cells. After a short-term exposure to colchicine, the frequency of the stem cells sharply dropped in the recovering population. After sustained colchicine action, a population was recovered which was entirely different, both in chromosomal characteristics and in growth potentiality, from the original one. This work shows that relationship between genetic differences and physiological characteristics can be demonstrated if a suitable material is found.

In populations such as strain L, where hundreds of cell types coexist in a seemingly harmonious manner, it would not be unreasonable to believe that there are metabolic interplays to maintain a delicate equilibrium. The situation may be similar to the changes of inversion sequences recorded in the natural populations of *Drosophila pseudoōbscura*, which are cyclic responses to ecological changes. However, we occasionally observe directional changes of population structure without any deliberate application of agent or agents. This observation suggests that even differences in culture-handling, such as changing to a new medium formula or alteration of subculture schedule, may act as forces that cause new adaptation. Such change was reported by Hsu and Kellogg (*5*) when they isolated the subline L-P59 from L-P55. Strain L-P59 was actually a spare stock of the original L-P55, then carried in Eagle's medium. In approximately 2 years, the spare population lost an average of 4 chromosomes per cell.

It appeared to us that the most likely difference between these two stocks was that L-P55 was subcultured approximately once a week to maintain a large supply, whereas L-P59 was kept most of the time at its plateau growth phase until subculturing was necessary. To demonstrate that routine handling of cultures can exert effect on population structure, the present article, using chromosomal characteristics as criteria, describes the results of some simple experiments to elucidate the balance as well as competition among cell types.

MATERIALS AND METHODS

Strain L-P59, a subline of strain L of the mouse, was used in this study. The history of this strain, culture methods, and cytological techniques were described in our previous paper (*12*).

RESULTS

Chromosomes of L-P59

For convenience in describing experimental results, some pertinent cytological characteristics of strain L-P59 are reviewed. Strain L-P59 had a stemline number of 63 to 65 chromosomes, among which 12 to 13 were metacentrics, 3 were subtelocentrics, and 1 was known as the D chromosome. The D chromosome was a long, subtelocentric element with a deep, secondary constriction near the terminal portion of the long arm. Most of the cells contained a single D chromosome (fig. 2), but cells with no such chromosome (fig. 1) or with 2 such chromosomes (fig. 3) occurred at a low frequency.

Competition Between Single Stem Cells and Double Stem Cells

A derivative of strain L-P59, known as strain Amy, was established by Hsu and Kellogg after long-term colchicine treatment to populations of L-P59 (*12*). Strain Amy had an average of 128 chromosomes per cell among which 2 were D chromosomes. When strains L-P59 and Amy were tested individually for their growth potentialities under identical cultural conditions and initial cell inoculums, strain Amy grew to approximately half the total cell number attained by L-P59. It must be added, however, that if total deoxyribonucleic acid (DNA) and protein were measured, endpoints of these two lines were identical. Thus, to multiply once, an Amy cell would have to synthesize twice as many cellular components as would an L-P59 cell. In other words, L-P59 cells were twice as "efficient" as Amy cells. If equal numbers of these 2 cell types were placed in a container to form a mixed population and no interaction between them existed, L-P59 cells would be expected to dominate the population in a relatively short time.

To each Owen's Oval bottle (2 oz. size), 5×10^5 L-P59 and the same number of Amy cells were inoculated in 5 ml of growth medium. No feeding was made after the cultures were set. Beginning day 2, samples were taken every other day to determine the proportion of L-P59 and Amy cells among the cells in mitosis. It must be mentioned that approximately 1 to 3 percent of L-P59 cells were double stem cells, which were indistinguishable from Amy cells. Thus, the true value of the Amy cells should be slightly lower than indicated in tables 1 and 2.

Table 1 represents the results obtained from the experiment just described. The percentage of L-P59 cells and Amy cells was approximately equal at day 2, and maintained a 60 : 40 value until day 8, at which time Amy cells in mitosis dropped to 23 percent. On day 12 the proportion of Amy cells in mitosis was reduced to approximately 16 percent.

TABLE 1.—Percentage of L-P59 and Amy cells in mitosis in mixed populations. Original inoculum, 5×10^5 cells in 5 ml of growth medium. No feeding was made after cultures were set

Age of culture (*days*)	L-P59	Amy	Number of cells
2	50. 9	49. 1	228
4	57. 7	42. 3	362
6	59. 8	40. 2	368
8	76. 4	23. 6	508
10	82. 8	17. 2	326
12	84. 1	15. 9	126

The great difference between the number of L-P59 and Amy cells when cultures grew old (table 1) was probably due to two factors: the difference in growth potentiality in the early period and the difference in the capacity of entering mitosis at later stages. In other words, the proportion of mitotic cells of a particular subcomponent in a mixed population is a function of two possibly independent variables: mitotic rate and absolute number of cells. For 12 days of competition, there is no reason to suppose that Amy cells were already reduced from the original 50 to 16 percent. To determine whether L-P59 cells could eventually overtake the entire population, 2 identical sets were set up, each represented by 1 T-60 flask which contained 2×10^6 each of L-P59 and Amy cells in 20 ml of growth medium. Without further feedings, they were allowed to grow for 7 days, at which time the cultures were trypsinized and subcultured. From each subculture, only 4×10^6 cells were used to initiate new growth, and the surplus cells were either cultivated separately for cytological checkup or discarded. Cultures for chromosomal studies were invariably fixed on their 4th day of incubation. Table 2 represents the recordings of these 2 series of experiments covering 4 weeks. In both series the proportion of Amy cells—which also included a fraction of double stem cells present in L-P59—dropped from the original 50 percent to 11 to 12 percent in 2 weeks and to 4 or 5 percent in 4 weeks, which was close to the value of double stem cells in ordinary L-P59 populations.

TABLE 2.—Reduction of Amy cells in mixed populations with L-P59. Initial inoculum, 2×10^6 cells each in 20 ml of growth medium. Every 7 days the cultures were trypsinized, from which 4×10^6 cells were recultured. The surplus cells were either used for cytological studies (data for this table) or discarded. Data for this table were collected from 4-day old cultures

Age of culture *(weeks)*	Series A		Series B	
	% Amy cells	Number of cells	%Amy cells	Number of cells
2	12. 5	327	11. 8	340
4	5. 1	428	2. 8	535

Competition Between Stem Cells and Slow-Growing Variant Cells

A slow-growing variant of L-P59, strain Barbara, was established at the same time as strain Amy from L-P59 after long-term colchicine treatment (*12*). With a given amount of medium and inoculum, Barbara cells could grow to only one fourth or, at the maximum, one third the cell number reached by strain L-P59. Barbara cells had a mode of 58 to 59 chromosomes per cell without the D element characteristic of L-P59. Instead, they had a large, conspicuous metacentric element as their marker. The presence of the D or the large metacentric (known as the Victoria chromosome) was used to identify these two cell types in mixed populations. As with distinguishing L-P59 and Amy, some error was unavoidable, because a small fraction of L-P59 cells did not possess the D chromosome, and some of them even possessed a large metacentric similar to the size of Victoria. However, very few Barbara cells did not possess the Victoria element. Thus, in recording the cells from mixed populations, there was a tendency, however slight, to increase the proportion of Barbara cells.

To witness the competition between these 2 cell lines, experiments identical with those containing L-P59 and Amy lines were set up, except that Amy cells were replaced by Barbara cells. Tables 3 and 4 show that Barbara cells were not able to compete with L-P59 cells as expected. However, it was a mild surprise to see that Barbara cells resisted elimination better than Amy cells did (*cf.* tables 1 and 2).

TABLE 3.—Percentage of L-P59 and Barbara cells in mitosis in mixed populations. Original inoculum, 5×10^5 cells in 5 ml of growth medium. No feeding made after cultures were set

Age of culture *(days)*	L-P59	Barbara	Number of cells
6	74. 0	26. 0	215
8	64. 0	35. 9	320
10	79. 0	29. 0	255
12	66. 4	23. 6	161

TABLE 4.—Reduction of Barbara cells in mixed populations with L-P59. Initial inoculum, 2×10^5 cells each in 20 ml of growth medium. Every 7 days the cultures were trypsinized, from which 4×10^5 cells were recultured. The surplus cells were either used for cytological studies (data for this table) or discarded. Data for this table were collected from 4-day old cultures

Age of culture (*weeks*)	Series A		Series B	
	% Barbara cells	Number of cells	% Barbara cells	Number of cells
4	10.0	144	20.5	170
6	4.2	71	11.9	132
11	4.2	311	4.0	322

Age of Culture as Selecting Factor for Genomic Adaptation

To detect whether there were any karyotypic differences between the mitotic cells at earlier stages of culture life and those at later stages, cultures of L-P59 and of Barbara were set up separately in Owen's Ovals containing 5 ml of growth medium each. Initial inoculum was 5×10^5 cells. No medium feeding was made after the cultures were set. The following criteria were at first used in analysis:

(1) Percentage of polyploids (double stem cells and higher)
(2) Total chromosome number per cell
(3) Number of biarmed chromosomes, including metacentrics and subtelocentrics (M + S) per cell
(4) Number of D chromosomes per cell

Samples were taken every other day, beginning at day 2. Table 5 presents the percentage values of polyploid cells of the 2 sublines recorded, with a minimum of 200 cells per sample. In some samples, cells observed exceeded 500. It was somewhat of a surprise to see that 16-day-old cultures of L-P59 without feeding still contained a few cells in mitosis. In Barbara the percentage of polyploids varied from 3.3 to 5.8; and in L-P59, from 0.6 to 3.6. In both there seemed to be a rise of the percentage of polyploids between days 6 and 8.

TABLE 5.—Percentage of double stem cells in populations of L-P59 and Barbara; minimum cell number for each population, 200

Age of culture (*days*):	2	4	6	8*	10	12	14	16
Strain L-P59	1.0	1.1	2.1	*3.6*	1.7	1.1	1.7	0.6
Strain Barbara	—	4.5	5.2	*5.8*	3.3	3.3	—	—

* Italics = highest proportion of double stem cells found in all samples.

Table 6 summarizes data on total chromosome number, the M + S chromosomes, and those of the D chromosome per cell in the stemline zone. Because of a greater labor in analyzing all these details than in recording proportions between stem cells and polyploids, each sample consisted of approximately 25 cells. The first 2 categories did not show

TABLE 6.—Mean total chromosomes, metacentric and subtelocentric chromosomes (M + S), and D chromosomes per cell in populations of L-P59 and Barbara. Each sample was comprised of 25 ± cells in stemline zone—polyploid cells excluded

Age of culture (*days*):		2	4	6	8	10	12	14	16
Strain L-P59	Total chromosomes	62. 00	64. 48	63. 16	61. 50	62. 20	62. 88	63. 42	60. 92
	M + S chromosomes	15. 68	15. 08	15. 04	15. 21	15. 54	14. 16	14. 15	—
	D chromosomes	0. 96	0. 96	1. 16	0. 92	1. 08	1. 56	1. 50	2. 04
Strain Barbara	Total chromosomes	57. 23	58. 48	58. 84	58. 76	57. 60	58. 24	—	—
	M + S chromosomes	18. 26	18. 52	18. 12	17. 75	17. 80	17. 54	—	—
	D chromosomes	0. 00	0. 00	0. 00	0. 00	0. 00	0. 00	—	—

significant changes throughout, though there seemed to be a tendency to lower the average value of M + S chromosomes as the cultures aged. The most significant change was noted in the number of the D chromosomes per cell in strain L-P59. During the first 10 days the average number of D chromosomes per cell fluctuated around unity (range 0.92–1.08). Beginning day 12, however, mitotic cells containing 2 D chromosomes suddenly increased in number, changing the average value to 1.5. The most striking phenomenon was observed on day 16. In this sample every cell except 1 contained at least 2 D chromosomes. Some cells even contained 3 such elements (fig. 4), and in one particular cell not included in the table, 4 D chromosomes were observed.

This sudden rise of frequency of cells with 2 or more D chromosomes at late stages of culture life was confirmed by 2 additional sets of experiments. In these experiments only the D chromosomes were recorded. Table 7 represents the combined data from these 3 sets, giving percentage values for each type of cell in respect to the D chromosome and the average values.

Does Old Medium Contain Factors That Affect Cellular Activities?

It appears from data in table 7 that age of culture was an influential factor for cellular activities. Apparently cells with 0 or 1 D chromosome grew better in the early period of culture life, but subsided when the cultures aged. On the contrary, cells with 2 or more D chromosomes either were not affected by or became more active under the conditions existing in the old cultures. Did crowded conditions play a part in determining cellular activities? In tissue culture we are all aware of the fact that seeding below an optimum cell inoculum yields poor growth. Perhaps the reverse, being crowded, irrespective of medium conditions, also influences cellular activities. However, it is more likely that old, used medium was important in altering the adaptation of genotypes. Changes in *p*H, exhaustion of vital nutrients, accumulation of metabolic wastes, etc., should all come into consideration.

A simple experiment was designed to determine whether old medium could change the genomic picture of a young culture. Before the experiments were started, medium which had supported growth of L-P59 cells for 14 days was pooled, filtered, and stored at 4° C. This medium, henceforth referred to as used medium, because of the filtration process,

TABLE 7.—Percentage of cells with 0, 1, 2, and 3 D chromosomes in populations of L-P59—polyploid cells excluded

Age of culture (*days*):		2	4	6	8	10	12	14	16
Number of D chromosomes:	0	6.1	10.8	10.2	15.0	4.2	2.0	2.0	0.0
	1	75.7	73.9	74.6	73.8	83.3	44.0	50.0	4.0
	2	18.2	15.3	15.2	11.3	12.5	54.0	48.0	88.0
	3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0
Mean D chromosomes		1.12	1.04	1.05	1.04	1.08	1.52	1.47	2.04
Number of cells		132	157	177	80	24	50	50	25

TABLE 8.—Mean D chromosome values per cell from young populations of L-P59 fed for 3 days with used medium (grown L-P cells for 14 days) or fresh medium—polyploid cells excluded

Samples	Fresh medium		Used medium	
	D chromosomes	Number of cells	D chromosomes	Number of cells
1	1. 12	25	1. 52	25
2	1. 28	43	1. 73	37
3	1. 17	52	1. 72	43
4	1. 14	58	1. 78	50
5	—	—	1. 64	50
6*	—	—	1. 60	50

*pH of used medium readjusted to 7.2.

showed a rise of *p*H from the original 7.0, or thereabouts, to 7.6. Unless otherwise stated, no readjustment of the *p*H was made. From the regular L-P59 stock, cultures were then set up in Owen's Ovals, each with 5×10^5 cells as initial inoculum with 5 ml of fresh medium. All cultures were allowed to grow for 2 days and the medium discarded. To one set of cultures was introduced 5 ml of fresh medium, and to the other, the same amount of used medium per culture vessel. Both sets were then reincubated for 3 additional days, at which time the cultures were fixed for cytological examination.

A total of 4 separate sets was made. Two additional sets were initiated in the same manner without the fresh medium controls, but to 1 of them used medium with its *p*H readjusted to 7.2 was added. Table 8 summarizes the data collected from these experiments. No drastic alteration in the number of D chromosomes was recorded after fresh medium was fed, but a rise was noted after used medium—a D-chromosome frequency equivalent to that of cultures 14 days old. Since the total age of these cultures was only 5 days and the cultures were not crowded, denseness, therefore, did not contribute to the change. Furthermore, from 1 experiment it appeared that *p*H difference did not have a demonstrable effect on the frequency of the D chromosome.

Since the used medium was originally 14 days old, and it was used again for 3 additional days, the medium was 17 days old by the time the cultures were fixed. According to table 6, the mean D-chromosome value on day 16 was 2.04. The values listed in table 8 were thus below what would be expected from a 17-day-old medium. It is possible that some of the cells with 0 or 1 D chromosome had enough reserve material when the used medium was introduced so that they could continue to divide in spite of the used medium. However, it is not a very satisfactory explanation, because cells that had their DNA doubled would have entered division before the 3-day period ended.

Can a Population Be Compelled to Shift Its Composition by Changing Its Cultural Schedule?

When cells with 0 or 1 D chromosome diminished in number in older cultures, it is likely that the conditions were not favorable for their

reproduction. The lowered frequency of cells with 2 D chromosomes in young populations, however, does not necessarily suggest that such conditions were harmful to their growth and reproduction. Neither does it suggest that they were particularly suitable for growth in aged cultures. Our routine subculture system of once a week probably favored strongly the overpopulation of cells with 1 D chromosome, thus crowding out the genomes with 2 D chromosomes, which were not as powerful as the former in such an environment.

If the subculture system could be changed so that the conditions would favor the cells with 2 D chromosomes, we would expect a rise of these genomes even in younger populations. A culture of L-P59 was subcultured twice a week for 2 weeks to ensure uniformity of the D-chromosome content. The population was then divided into 3 subcultures, 1 of which was fixed on day 4 for cytological examination as the initial population. The second culture was subcultured twice a week from this point on, and the third, once in 2 weeks. In no case were the cultures fed between subcultures. From time to time samples were taken from these 2 stocks for analysis, invariably fixed on day 4. Table 9 presents the average values of D chromosomes per cell of the initial as well as the 2 stocks carried for 4 months.

TABLE 9.—Percentage of cells with 0, 1, 2, and 3 D chromosomes and mean D chromosome values in 2 sublines of L-P59, one being subcultured twice a week and the other, once in 2 weeks. All samples were taken on the 4th day of subculture—polyploid cells excluded

Subline	Age of culture (*weeks*)	Number of D chromosomes per cell				Mean D chromosomes	Number of cells
		0	1	2	3		
Initial	0	8.0	76.0	16.0	0.0	1.08	25
Twice a week	5	16.0	72.0	12.0	0.0	0.96	25
	13	12.2	74.4	13.4	0.0	1.01	82
	17	20.5	75.0	4.5	0.0	0.84	44
Once in 2 weeks	7	8.0	36.0	56.0	0.0	1.48	25
	11	5.4	27.0	67.6	0.0	1.62	37
	16	3.9	22.4	71.1	2.6	1.69	76

It is apparent that, in the stock which had been subcultured once in 2 weeks, cells with 2 D chromosomes greatly increased in number even in young populations. Since each sample was taken at day 4, it is equally evident that fresh-culture conditions did not impair the growth and reproductive activities of such cells.

DISCUSSION

Perhaps we should also consider it a "lucky break" when the most conspicuous chromosome of strain L-P59 showed differential response to cultural conditions. Had it been an obscure chromosome that was

involved, the analyses would have been either difficult, and consequently more inaccurate, or impossible.

The response of the D chromosome to cultural conditions described in this communication was, in fact, quite unexpected. Ironically, we had expected to see the reverse. Since colchicine treatment reduced the frequency of cells containing the D element, and since strain L-P55 reduced its average D chromosome value in the neglected stock which finally became strain L-P59 (*12*), it appeared reasonable at that time to regard cells without D chromosomes as inferior types, thereby entering mitosis at a later period of culture life. We still think that cells without D are not as vigorous as the stem cells, but it is apparent now that the lack of growth vigor does not necessarily mean that they must undergo mitosis when the culture is aged. They may still grow better in younger cultures, as the growth curve of strain Barbara indicated (*12*). The apparent contradiction between our present data on L-P59 and the observation of L-P55 cannot be fully explained. One difference, however, existed between the 2 populations, *viz.*, L-P55 had a mean chromosome number of 68, whereas L-P59 had 64 or lower. Whether the additional 4 or 5 chromosomes contributed to the reversing of cell physiology will remain a question.

The present report shows that different genomes do have different metabolic optima. An extremely vigorous one may be the best only so long as the conditions remain favorable. Otherwise it may become inferior and be replaced. In any cell strain where genetic heterogeneity is a rule, a cyclic adaptation system conceivably exists. After fresh subculture, some genomes become extremely vigorous, but their vigor subsides when conditions change. Other genomes then begin to be more active. In the experiments described in table 9, it is clear that a deliberate deviation of routine culture system may compel genotypes in the majority to be in the minority. Nevertheless, this was not a complete replacement.

In this connection we shall bring out again the ever-intriguing problem of the equilibrium of polyploids in cell populations. Data have shown that the double stem cell of L-P59 produced only half as many cells at the plateau growth phase (7 days) as the stem cells (*12*). If in the initial population equal numbers of these two genomes are placed in competition, after a weekly subculture system, the proportion of stem cells versus double stem cells would be expected to be as 2^n:1, respectively, where n denotes the number of subcultures. Thus the double stem cells should be below 0.1 percent in 10 and below 0.01 percent in 14 subcultures, or roughly 100 days. The actual situation was not so. It is indeed true that the double stem cells could not possibly compete with the stem cells, as evidenced by data in tables 1 and 2, but, for years of cultivation, the strain has maintained around 1 to 2 percent of double stem cells (table 5). Two possibilities exist: 1) The 2-s cells are constantly generated by the s cells, and 2) the 2-s cells have some adaptational values during a particular period of cultural cycle. Although the scanty data in table 5, which show a weak rise of polyploids on days 6 to 8, could hardly be treated

seriously, they are nevertheless an interesting lead. Working on porcine cell cultures, Ruddle (*13*) found a similar phenomenon in his system.

It is of interest to read our discussion of 2 years ago on the D chromosome, then regarded as a dicentric (*2*): "When there was a chance for them to acquire 2 such dicentrics, there should be a chance for them to acquire 3. The fact that all the cells possessed not more than 2 dicentrics indicates that the genic material in this chromosome was essential but could not be overdosed." Now we know that it could, except that the physiology of cells with 3 or more D elements was so particular that one rarely observes them on regular populations until a cell culture becomes very old. Attempts are being made to clone the L-P59 cells in order to recover lines relatively pure in their content of D chromosomes with which to compare their physiological characteristics.

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PLATE

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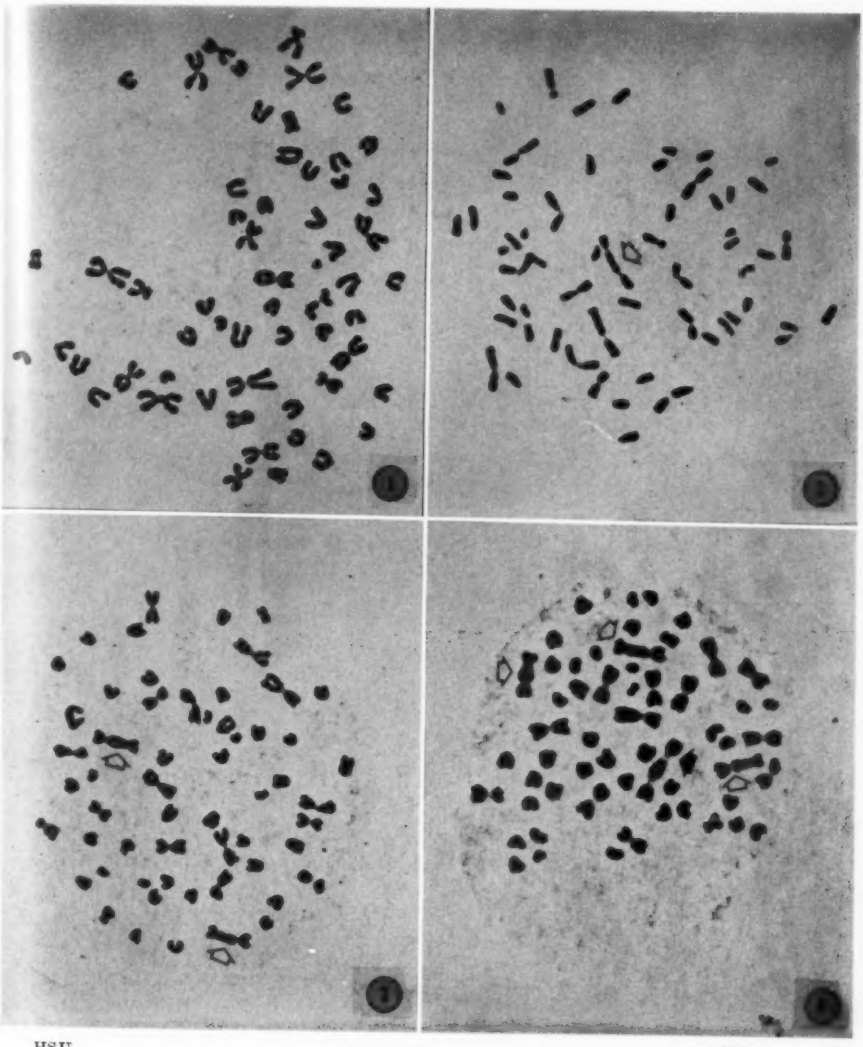
PLATE 175

FIGURE 1.—A cell of strain L-P59 without D chromosomes.

FIGURE 2.—A cell of strain L-P59 with 1 D chromosome (*arrow*).

FIGURE 3.—A cell of strain L-P59 with 2 D chromosomes (*arrows*).

FIGURE 4.—A cell of strain L-P59 with 3 D chromosomes (*open arrows*). The "dicentric" chromosome indicated by *solid arrow* is not a D chromosome and is not a necessary member for cells with 3 D chromosomes.



HSU 1353

Growth Inhibition of Chick-Embryo Cells by Some 5-Fluorinated Pyrimidines: Effect on Rous Sarcoma Virus Infection [1,2,3]

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SUMMARY

Except for a tenfold difference with 5-fluorouridine, the toxicities of several fluorinated pyrimidines and their corresponding nucleosides for secondary cultures of chick-embryo fibroblasts were similar to those previously reported for human tumor and other mammalian cell lines. Reversal patterns with pyrimidine metabolites were similar for 5-fluoro-2′-deoxyuridine and 5-fluoro-2′-deoxycytidine, but different with 5-fluorouracil and 5-fluorouridine. Reversal of 5-fluoro-2′-deoxyuridine inhibition by deoxyuridine appeared to be of the competitive type, while the reversal by thymidine suggested noncompetitive reversal. An approximately 15-hour period during which complete growth inhibition could be reversed by transfer to thymidine-supplemented medium was noted. Incubation of chick-embryo fibroblasts in 5-fluoro-2′-deoxyuridine for 18 hours before or after exposure to Rous virus had no significant effect on their capacity to be transformed to sarcoma cells, as measured by the appearance of foci of transformed cells in the *in vitro* assay procedure.—J. Nat. Cancer Inst. 25: 1355–1363, 1960.

THE 5-FLUORINATED pyrimidines and their corresponding nucleosides (*1–3*) have received considerable attention during recent years. Studies on the mechanism of action of 5-fluoro-2′-deoxyuridine and 5-fluoro-2′-deoxycytidine in mammalian systems suggest a specific interference with deoxyribonucleic acid (DNA) synthesis (*4–9*), probably at the level of thymidylate synthetase (*10*). In view of the use of chick-embryo fibroblasts as a host for various viruses and of our own interest in the Rous sarcoma virus-chick fibroblast system as a model for the study of tumor virus-host cell relationships, it was of interest to extend our studies on the 5-fluorinated pyrimidines to chick-embryo fibroblasts in cell culture.

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[3] Presented in part at annual meetings of the Society of American Bacteriologists, May, 1960, Philadelphia, Penna.

[4] The authors wish to acknowledge the assistance of Miriam Black and Amaury Perez. We are indebted to Dr. H. Rubin for his helpful suggestions concerning the Rous virus assay.

Time and concentration parameters for inhibition and subsequent reversal of growth inhibition by 5-fluorouracil, 5-fluorouridine, 5-fluoro-2′-deoxyuridine, and 5-fluoro-2′-deoxycytidine were studied.

The effect of 5-fluoro-2′-deoxyuridine on the capacity of chick-embryo fibroblasts to be transformed to sarcoma cells by Rous virus was studied with the *in vitro* assay developed by Temin and Rubin (*11*).

MATERIALS AND METHODS

Compounds.—5-Fluorouracil (FU), 5-fluorouridine (FUR), and 5-fluoro-2′-deoxyuridine (FUDR) were synthesized by Hoffman-La Roche, Inc. (*3*). The 5-fluoro-2′-deoxycytidine (FCDR) was synthesized by Fox, Wempen, and Duschinsky (*2*). Other compounds were purchased from the California Corporation for Biochemical Research.

Culture methods.—The culture medium, a modification of Eagle's medium with double strength amino acids and vitamins and supplemented with 10 percent calf serum, was used throughout and will be referred to as RC_{10}.

The eviscerated trunks of chick embryos, aseptically removed from 10-day White Leghorn eggs (Shamrock Farms), were minced with scalpels and transferred to a trypsinizing flask. They were washed with saline A (*12*), and trypsinization with 0.05 percent trypsin (Difco 1 : 250) was carried out at 37° C for 5 minutes. The resulting washed-cell suspensions were added to 60 ml of RC_{10} in 1-liter, screw-capped bottles. Incubation in a humidified incubator, flushed with a CO_2-air mixture, was continued for 3 days. Primary monolayers were washed and cell suspensions obtained by trypsinization. Cells, 2 to 3 $\times$ 10^5, were added to 4 ml of RC_{10} in 60 mm petri plates. After overnight incubation (to allow cells to attach to glass) the medium was replaced and the fluoro-pyrimidines and/or metabolites were added. Incubation was continued for 6 days, with change of medium and compounds at 3 days. After triple washing to remove medium and nonadhering cells, growth measured as total protein was determined by the colorimetric method of Oyama and Eagle (*13*). To determine the degree of inhibition and/or reversal, growth in control plates was taken as 100 percent with protein values, at time of addition of compounds, representing zero growth. Values given represent the mean of triplicate plates, and experiments were carried out at least twice.

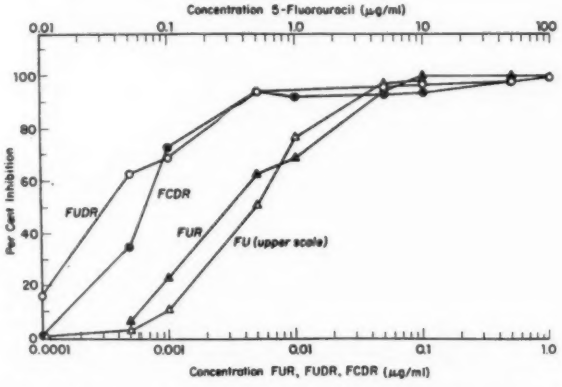
Viral studies.—Partially purified stocks of Rous sarcoma virus (Lot #CT–842), generously supplied by Dr. W. R. Bryan of the National Cancer Institute, were stored in sealed glass ampoules in a dry-ice chest. Ampoules were thawed and diluted in RC_{10} medium.

These studies employed a modification of the *in vitro* assay for Rous sarcoma virus and Rous sarcoma cells described by Temin and Rubin (*11*). Approximately 5 $\times$ 10^5 cells prepared from the primary monolayers by trypsinization were added to 60 mm "flat-bottom" petri plates (Bellco Glass Co.) containing 4 ml RC_{10} and allowed to attach overnight.

Monolayers were exposed to 0.5 ml of the appropriate dilutions of virus (in RC_{10}) for 1 hour at 37° C. Where appropriate, FUDR was added to the medium before, during, or after exposure to virus. Virus plates were overlaid with RC_{10} containing 0.9 percent agar. After incubation for 1 week, plates were examined for the presence of characteristic foci of transformed Rous sarcoma cells (*11*).

RESULTS

The comparative toxicities of FU, FUR, FUDR, and FCDR are shown in text-figure 1. It is evident that all the antimetabolites tested were potent growth inhibitors of chick-embryo fibroblasts. FU, which demonstrated essentially complete growth inhibition at 5 μg/ml was approximately 100-fold less toxic than FUR and 1,000 times less toxic than the deoxyribosides FUDR and FCDR.



TEXT-FIGURE 1.—Growth inhibition of chick-embryo fibroblasts by 5-fluorouracil (FU), 5-fluorouridine (FUR), 5-fluro-2′-deoxyuridine (FUDR), and 5-fluoro-2′-deoxycytidine (FCDR).

Reversal

The ability of various pyrimidine metabolites to reverse growth inhibition by these fluorinated antimetabolites is shown in table 1. In all cases metabolites were added at the same time as the antimetabolites. The bases uracil, cytosine, and thymine were ineffective in reversing the action of any of the antimetabolites employed. At an inhibitor-to-reverser ratio of 1:100, FUR could be reversed by the ribosides uridine and cytidine, but not by the corresponding deoxyribosides, deoxyuridine, and deoxycytidine even at ratios of 1:500. Thymidine and 5-methyldeoxycytidine were also ineffective at this ratio. The reversal spectrum for FUDR and FCDR was identical. The fluorinated deoxyribosides were reversed by thymidine at ratios of 1:1 and by methyl-

TABLE 1.—Effect of several compounds on growth inhibition of chick-embryo fibroblasts by FUR, FUDR, and FCDR

Compound	Concentration	Reversal of inhibition*		
		FUR (0.1 μg/ml)	FUDR (0.1 μg/ml)	FCDR (0.1 μg/ml)
Thymine	50	−	−	−
Thymidine	0.1	−	+	+
"	1.0	−	+	+
"	10.0	−	+	+
Uracil	50	−	−	−
Uridine	10	+	−	
"	50	+	−	−
Deoxyuridine	5	−	+	+
"	50	−	+	+
Cytosine	50	−	−	−
Cytidine	10	+	−	
"	50	+	−	−
Deoxycytidine	50	−	−	−
Methyldeoxycytidine	1	−	+	+
"	10	−	+	+
"	50	−		

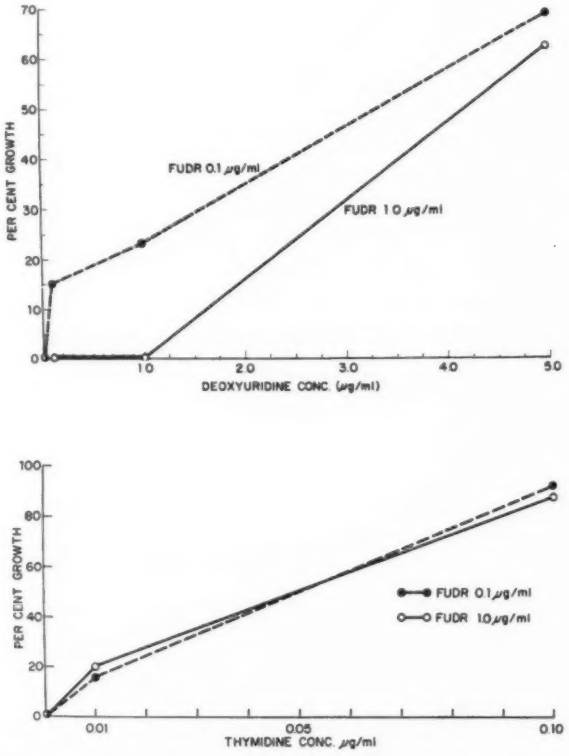
*+ = Significant reversal (>35%);
− = no significant reversal.

deoxycytidine at a 10 times higher concentration. While deoxyuridine at a ratio of 1:50 reversed growth inhibition by FUDR and FCDR, deoxycytidine did not reverse the action of either inhibitor even at a ratio of 1:500. The ribosides uridine and cytidine at 1:500 were similarly unable to reverse growth inhibition by either antimetabolite.

Since in previous studies (*4*) with mammalian cells both a competitive and noncompetitive reversal of growth inhibition could be demonstrated with deoxyuridine and thymidine, respectively, a similar study with chick fibroblasts was carried out. Growth inhibition at two levels of FUDR was determined in the presence of a series of concentrations of thymidine and deoxyuridine. With deoxyuridine (text-fig. 2), an increase in inhibitoconcentration from 0.1 to 1.0 μg/ml required an increase in the concentrar tion of deoxyuridine to give an equivalent degree of reversal. This dependence on the *ratio* of inhibitor to reverser suggested a competitive reversal. With thymidine (text-fig. 2), reversal appeared to depend only on the level of reverser. A given concentration of thymidine gave the same percent reversal independent of the level of FUDR present (0.1 or 1.0 μg/ml). This is indicative of noncompetitive reversal. Similar results were obtained with the reversal of FCDR inhibition by thymidine and deoxyuridine.

To utilize these compounds in viral studies, it was necessary to determine the time parameters for growth inhibition. Cells were grown in the

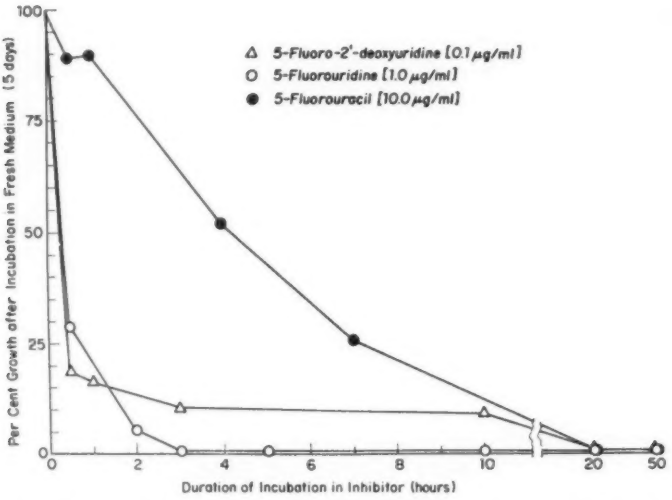
presence of FU, FUR, and FUDR for varying periods from ½ to 50 hours, at which time the medium was replaced with fresh inhibitor-free medium. Incubation was then continued for 5 days. The subsequent growth in the inhibitor-free medium was determined and is recorded in text-figure 3. Exposure to fluorouridine or fluorodeoxyuridine for as little as half an hour resulted in almost complete growth inhibition. With FU, growth



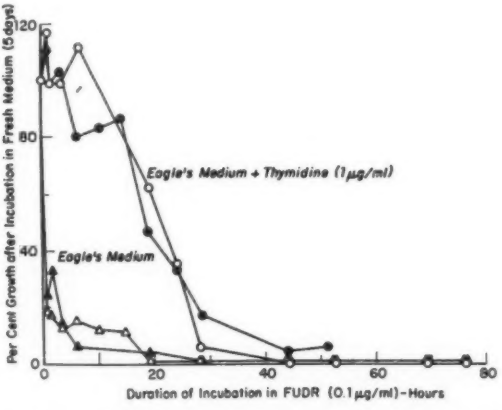
TEXT-FIGURE 2.—Effect of thymidine and deoxyuridine on the growth of chick-embryo fibroblasts in the presence of two concentrations of 5-fluoro-2′-deoxyuridine.

inhibition was more gradual. Fifty percent inhibition occurred at 4 hours, with complete inhibition at approximately 20 hours.

In another study with FUDR, cells were incubated in the presence of FUDR for from ½ hour to 75 hours, at which time the inhibitor was removed and replaced with fresh medium with and without thymidine. It can be seen from text-figure 4 that, although cells exposed to FUDR for as little as half an hour would not grow when transferred to unsupplemented medium, cells exposed to the inhibitor for as long as 15 hours would multiply if transferred to medium supplemented with thymidine.



TEXT-FIGURE 3.—Effect of incubation time in 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine on the subsequent growth of chick-embryo fibroblasts in inhibitor-free medium.

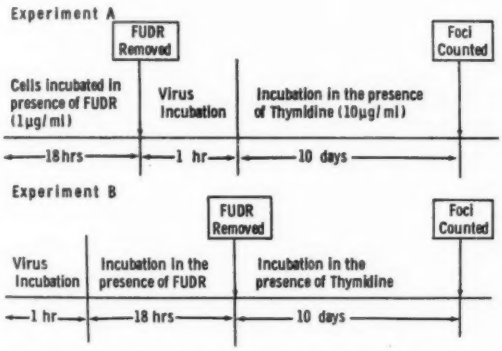


TEXT-FIGURE 4.—Effect of incubation time in 5-fluoro-2′-deoxyuridine on the subsequent growth of chick-embryo fibroblasts in an inhibitor-free medium in the presence and absence of thymidine.

Viral Studies

Transformation of chick-embryo fibroblasts to Rous sarcoma cells was assayed by the method of Temin and Rubin (*11*). Host cells were incubated in the presence of FUDR (1 μg/ml) for varying periods (1–18 hours) before, during, or after virus exposure. In all experiments thymidine (10 μg/ml) was added within 20 hours after the addition of FUDR,

to reverse growth inhibition and allow cells to grow. The protocol for a typical experiment appears in text-figure 5. Incubation in FUDR for 18 hours before or after virus exposure had no significant effect on the subsequent development of foci of transformed sarcoma cells (table 2). Other experiments with shorter periods of exposure to FUDR gave essentially the same results.



TEXT-FIGURE 5.—Typical protocol for experiments designed to determine the effect of exposure to 5-fluoro-2′-deoxyuridine on the capacity of chick-embryo fibroblasts to be transformed to sarcoma cells by Rous virus.

TABLE 2.—Effect of FUDR on the occurrence of foci of Rous sarcoma cells

Treatment (*18 hrs.*)	Number of foci ($\bar{x} \pm s$)	
	Control	FUDR
A. FUDR *before* exposure to virus	128 ± 8	119 ± 4
B. FUDR *after* exposure to virus	233 ± 14	216 ± 34

DISCUSSION

The inhibition and reversal patterns described are similar to those previously reported for mammalian-cell systems. However, the tenfold difference in toxicity between FUR and FUDR seen in text-figure 1 was not observed for H.Ep. #1 cells (*4*). The inability to reverse growth inhibition by FU with any of the metabolites used was in agreement with results for most of the mammalian lines studied (*14*). The apparent specificity for the reversal of fluorinated deoxyribosides by deoxyuridine and FUR by uridine and cytidine was also in agreement with results previously reported for mammalian cells (*4, 15*).

The ability of thymine-containing metabolites to reverse growth inhibition of chick-embryo fibroblasts supports the hypothesis, suggested by earlier work in mammalian cells grown in cell culture, that the principal site of action of FUDR and FCDR appears to be an interference with the synthesis of the thymine moiety of DNA (*4, 15*). As was

suggested for mammalian cells, FU and FUR probably interfere at sites in addition to those concerned with the conversion of deoxyuridylic to thymidylic acid (*4*). Conversion to ribonucleotides containing the fluorouracil moiety and the incorporation of this moiety into RNA have been demonstrated (*1*).

The approximately 15-hour "time effect" shown in text-figure 4, which suggests a manifestation of unbalanced growth (*5*), provides a convenient system for studying the effects of FUDR on the viral susceptibility of these cells to the Rous agent. It can be seen that, within half an hour after the addition of FUDR, growth inhibition of essentially the whole population had occurred. The fact that this inhibition could be completely reversed, if thymidine were added within approximately 15 hours, was particularly suited to a system in which infection is expressed by a proliferative infective center (as opposed to a lytic plaque).

It is evident from table 2 that cells, whose synthesis of DNA had been blocked, still retained their capacity to be transformed to sarcoma cells by the Rous virus. Further studies may elucidate the possibility that only the initial stages of the infective process can occur in the absence of DNA synthesis. The later steps leading to virus proliferation and subsequent transformation to sarcoma cells may occur *after* the resumption of DNA synthesis following the addition of thymidine.

Fairman (*16*) and Rubin and Temin (*17*), using the fluorinated base FU, found no effect on the development of foci of Rous sarcoma cells.

In view of the inability of FUDR, an inhibitor of DNA synthesis, to interfere with the transformation of chick-embryo cells by the Rous sarcoma agent, an RNA-containing virus, it is of interest to note the recent results with vaccinia and polio virus. Salzman reported that the production of vaccinia, a DNA-containing virus, was inhibited by FUDR, while the formation of RNA-containing polio virus was unaffected (*18*).

Defendi and Kritchevsky (*19*), using RPL-12 virus grown in chick-embryo liver cells, found that FUDR decreased virus production and reduced the number of intranuclear inclusions.

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Analysis of Enzymatic Activities of Clones Derived From Variant Cell Lines Transformed to Malignant Cells in Tissue Culture [1]

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SUMMARY

Two cell lines, derived *in vitro* from one cell isolated from a culture of normal mouse tissue, were found in previous studies to differ in morphology, metabolism, and rate of growth to form malignant tumors in mice of the strain of origin (C3H). When cells of one line, designated low tumor-producing or "low," were injected into mice, tumors developed slowly or not at all, whereas tumors developed rapidly from implants of the other cell line, designated high tumor-producing or "high." Three clones established from single cells of the low line and one clone from the high line have been investigated to determine whether there is any correlation between activities of some enzymes metabolizing glucose oxidatively and the rate of growth into tumors when cells are implanted in mice. The activities of the enzymes hexokinase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase have been determined in the whole cells and in cell-free extracts of the cells. The mean specific activities of all 3 enzymes were very similar for 2 of the clones from the low line. The third of the clones from the low line that differed in certain other characteristics had the same phosphogluconate activity, a 50 percent higher hexokinase activity, and twice the glucose-6-phosphate dehydrogenase activity. The clone derived from the high line had nearly 3 times the hexokinase activity, 4 times the glucose-6-phosphate dehydrogenase activity, and 5 times the phosphogluconate dehydrogenase activity compared with 2 of the low-line clones. The differences between the derivative clones from low and high lines are even greater when the enzyme activities of the whole cells or the extracts are calculated per cell.—J. Nat. Cancer Inst. 25: 1365–1379, 1960.

VARIANT LINES of tissue-culture cells arose *in vitro* from one cell isolated from a culture of normal subcutaneous connective tissue taken from a strain C3H mouse (*1*, *2*). Cells of some lines grew as malignant tumors in a high proportion of hosts when the cells were inoculated into C3H mice. Cells of other lines produced tumors infrequently or not at all. The characteristics of 2 lines, a "low" and a "high" tumor-producing line, have been studied extensively (*3*). In 1956, 3 clones were grown from single cells of the low tumor-producing line. These clones were

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designated NCTC 2445, 2470, and 2555. Clone 2555 was derived from a single cell, the nucleus of which divided and then appeared to fuse again (*4*). Clone NCTC 2472 was derived from a cell of the high tumor-producing line. To establish the clones, it was necessary to change the type of culture fluid from that used to originate and maintain the cell lines *in vitro*. Growth in the new medium, a chemically defined medium with serum supplement, so enhanced the transplantability of the cells that those of the original low line now grew progressively in almost all mice injected. However, the latent period for tumor formation in mice [3] was significantly longer for cells of the low line and all derived clones than for cells of the high line and its clone. Low-line clone 2555 failed to produce any tumors unless the host had been X irradiated, and in irradiated hosts the tumors developed slowly. Thus, the latent period for tumor formation *in vivo* appeared to be a heritable characteristic distinguishing cells of the 2 lines. Although the type of culture medium altered the transplantability of the cells, the original 2 cell lines will still be referred to in this paper as the "low" and "high" lines.[4]

The chromosomal constitution of cells of these clones and the lines of origin have been reported by Chu and associates (*5*). The modal chromosome number for cells of the low-line NCTC 2049 was 49, for clone 2445, 53, and for clone 2555, 69. Clone 2470 contained a high proportion of cells (37 %) with chromosome numbers higher than tetraploid ($2n = 80$). The modal chromosome number for the high-line NCTC 1742 appeared to be 56. Clone 2472 showed a slightly lower mode but an increased proportion of polyploid cells. Metacentric chromosomes were present in all lines and clones, whereas mouse chromosomes are normally telocentric.

The rates of anaerobic glycolysis of the clones have been investigated by Woods and associates (*6*) and found to be higher for clone 2472 than for the low-line clones. The activities of the enzymes arginase and rhodanese in cells of the low and high lines were reported by Westfall *et al.* (*7*). Activities were low and did not differ significantly between the two lines.

We are reporting studies on another series of enzymes, those dealing with glucose by the oxidative route, hexokinase, glucose-6-phosphate dehydrogenase (G-6-PD), and phosphogluconate dehydrogenase (PGD). The enzyme activities of the whole cells in suspension and of cell-free extracts have been correlated with tumor latent periods *in vivo* for clones 2445, 2470, and 2555 from the low line and for clone 2472 from the high line (*8*).

MATERIALS AND METHODS

Culture procedure.—The cells were cultured, as described previously (*4*), in chemically defined medium NCTC 109 supplemented with 10 percent

[3] The tumor latent period measured the interval from injection of cells to the appearance of a palpable tumor at the injection site that continued progressive growth ultimately diagnosed as a sarcoma.

[4] Low and high tumor-producing lines were originally referred to as lines of substrains III and VII, respectively (*1*). As carried in the laboratory, these lines are designated NCTC strains 2049 and 1742, and strains cultured in the serum-supplemented, chemically defined medium as NCTC strains 2643 and 2642, respectively.

horse serum (*9*, *10*). Fluids were renewed 3 times a week, when the cultures were gassed with a humidified mixture of 5 percent CO_2 in air to adjust the *p*H. Flasks were sealed with silicone stoppers. Five sets of cultures of each of the 4 clones were transported from Bethesda to Philadelphia in the flasks in which they had been growing—one set in May, 1958, two sets in June, 1958, and two sets in October, 1958. During transit, the temperature was kept as constant as possible by careful insulation and bottles of warm water. Two of the sets were incubated for 2 more days in Philadelphia and had continued to increase in numbers.

Preparation of cell suspensions and extracts.—To harvest the cells in the culture fluid in which they were growing, we gently swabbed the floor of the flask with a strip of perforated cellophane manipulated by a glass rod. The cell suspensions from 6 or 7 T-60 flasks of each clone were pooled, transferred to centrifuge tubes, and centrifuged at 500 rpm ($60 \times g$) for 10 minutes in a #2 International refrigerated centrifuge. The cell pellet was resuspended in a tared tube in a minimum (approx. 1.5–3 ml) of culture fluid and the volume brought to 6 or 7 ml with 0.4 M sucrose solution. Two samples of the agitated cell suspension, 0.1 ml each, were added to 0.1 M citric acid solution for enumeration of cell nuclei (*11*). Another sample, 0.4 or 0.5 ml, was removed for analysis of enzymatic activity of the whole cells. The remaining suspension was again centrifuged at 500 rpm for 10 minutes. The supernatant fluid was removed and the wet weight determined. The cells were ground at 2° C for 2 minutes with an amount of Al_2O_3 2.5 times the wet weight of cells (*12*) and extracted with 5 volumes of cold 0.4 M sucrose solution. This mixture was centrifuged at $2200 \times g$ for 60 to 70 minutes (first 3 sets of cells) or at $10,000 \times g$ for 60 minutes (last 2 sets of cells). No effect of the different speeds of centrifugation on dehydrogenase activities could be detected, though the low-speed extracts would contain more subcellular particles. On the other hand, the hexokinase activities of extracts of clones 2472, 2445, and 2555 in the last 2 sets were higher than those of the first 3 sets. The protein content of the extracts was determined by the bromosulphalein method of Bonting and Jones (*13*).

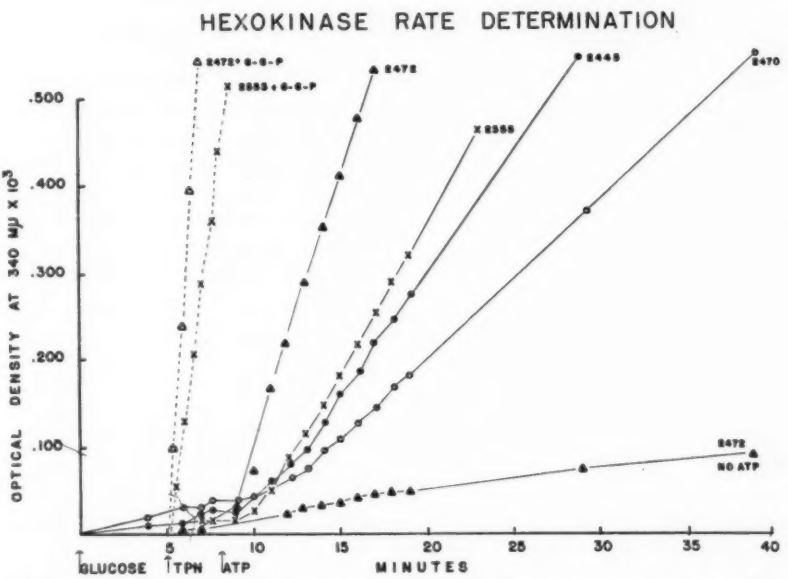
For comparison of these clones with the tissue of origin, 0.642 mg of connective tissue, primarily adipose, of the lateral subcutaneous fat pad was taken from 6 C3H mice. The lymph nodes were removed. The tissue was cut with scissors, ground with alumina, and extracted with 3.0 ml of 0.4 M sucrose. The mixture was centrifuged at $10,000 \times g$ for 1 hour. The extract contained 0.87 mg of protein/ml.

Determination of enzyme activities.—Enzyme activities were determined immediately on extracts kept in an ice bath, for the dehydrogenases are labile either on warming or freezing. The dehydrogenase activities were determined as rate of reduction of triphosphopyridine nucleotide (TPN) measured by the increase of absorption at 340 mμ in the Beckman DU spectrophotometer, which had a thermospacer to equilibrate the temperature at 35° C (*14*, *15*). Samples of the extract containing 50 to 300 μg of protein were added to 1.5 ml cuvettes containing tris buffer 0.05 M at *p*H

7.4 and substrate, either glucose-6-phosphate (G-6-P) 0.6 μmole, or phosphogluconate (PG) 0.3 μmole. TPN, 0.2 μmole in 0.02 ml, was added and readings taken at half-minute intervals for 5 minutes. In this sample range the rates of TPN reduction were proportional to the protein concentrations. The G-6-P dehydrogenase results were not corrected for reduction of TPN by PG dehydrogenase acting on the PG formed, for the correction was found to be negligible in the first 5 minutes, if the samples were chosen to give an increase of optical density of 0.010 to 0.050 per minute. The reduction of TPN by endogenous substrate was almost complete in the 1st minute. Therefore, calculations of rate were determined by the increase in optical density between 1 and 3 or 4 minutes without correction. Under these conditions there was no evidence of reduced triphosphopyridine nucleotide (TPNH) oxidase activity without the addition of oxidized glutathione.

Hexokinase was determined by a modification of the method of Slein, Cori, and Cori (*16*, *17*) as the rate of reduction of TPN caused by the endogenous G-6-PD and the G-6-P produced by hexokinase action on glucose + adenosine triphosphate (ATP). Samples of the extracts containing 150 to 600 μg of protein were added to 1 ml of solution of tris buffer 0.04 M, containing 1.1 μmoles of glucose and 1 μmole of $MgSO_4$. TPN, 0.4 μmole, was added and 5 minutes later, after the endogenous reaction was minimal, 1 μmole of ATP was added. The optical density at 340 mμ was read at minute intervals. The maximum rate attained was taken for calculation of hexokinase activity. In text-figure 1 are plotted a hexokinase reaction and the slow endogenous reaction in the absence of ATP. In these hexokinase determinations the endogenous G-6-P dehydrogenase was sufficient not to be rate-limiting, and there was no evidence of TPNH oxidase activity. The Michaelis constants for an extract of cells of clone 2472 were found to be approximately $K_{ATP} = 2.2 \times 10^{-4}$ and $K_{glucose} = 1.5$ to 3.0×10^{-5}. The rate was proportional to the concentration of protein at concentrations higher than 150 μg/ml of extracts of clone 2472, and higher than 400 μg/ml of clone 2555. The effect of activators and inhibitors of the hexokinase was not investigated with these extracts.

The glucose disappearance during the reaction was also determined by the notatin procedure of "Glucostat" (Worthington Biochemical Corp., Freehold, N.J.). For the most active extracts, those of clone 2472 cells, the correspondence was fair at times. For example, 0.36 μmole of glucose disappeared in 60 minutes or 0.006 μmole/minute, and the maximum rate of TPN reduction was 0.005 μmole/minute. For most of the determinations on glucose disappearance, the correspondence was not as good, more glucose disappearing than accounted for by the maximum rate. We must conclude, therefore, that the hexokinase determination has not given the optimal rate. Determination of the hexokinase rates of the different clones allows comparison, but cannot be considered to give absolute values, even for the extracts. Hexokinase activity in the particulate fractions was not determined.



TEXT-FIGURE 1.—A 1.5 ml silica cuvette contained 0.2 ml tris buffer, pH 7.4, 0.25 M glucose, 1.1 μmoles, $MgSO_4$, 1.0 μmole, and glass-distilled water to bring the final volume to 1 ml. The cuvettes were equilibrated for 10 minutes in the Beckman DU thermospacer at 35° C. At time 0, a sample of cell-free extract of tissue-culture cells of set IV, as indicated below, was added. The optical density was read at 340, 260, and 290 and again at 340 mμ. At 5 minutes, TPN, approximately 0.2 μmole, and at 10 minutes, ATP, 1.0 μmole, were added. The plotted readings are minus the initial background absorbence of the extracts and are designated as follows:

2445 ●—● 0.04 ml of extract of clone 2445 containing 0.436 mg protein
2470 ○—○ 0.04 ml of " " " 2470 " 0.352 mg "
2555 X—X 0.06 ml of " " " 2555 " 0.450 mg "
2472 ▲—▲ 0.05 ml of " " " 2472 " 0.280 mg "
2472-A, no ATP was added
2472 and 2555 + G-6-P had 0.3 μmole of G-6-P, no glucose, and no ATP.

Whole-cell enzyme activities were determined with 0.05 or 0.1 ml of cells in suspension in the same way as with extracts, except that the reaction mixture was stirred before each set of readings to insure a uniform suspension. G-6-P and 6-PG were obtained as barium and sodium salts from Sigma Chemical Company, St. Louis, Missouri. TPN and TPNH were obtained from Sigma and from Pabst Company, Milwaukee, Wisconsin, and were approximately 96 percent pure.

RESULTS

The counts of nuclei per ml of suspension are given in table 1. The protein extracted per cell was calculated as shown in table 2. If 15

TABLE 1.—Count of nuclei in suspensions

Clone No./set of cultures	Nuclei/ml × 10^{-6}				
	I	II	III	IV	V
2470	31. 5	11. 1	8. 7	19. 0	19. 0
2445	15. 6	15. 0	15. 0	40. 0	49. 0
2555	24. 1	12. 3	16. 8	25. 0	27. 0
2472	13. 6	7. 9	0. 9	10. 0	6. 0

TABLE 2.—Protein of cell-free extracts

Clone No./set of cultures	mg/10^9 cells			
	II	III	IV	V
2470	235	520	150	155
2445	151	155	150	100
2555	152	270	166	133
2472	300	1150	180	200

percent of the wet weight of the cells is assumed to be protein, the extracted protein, not sedimented, represents about 33 percent of the total cell protein.

Except for the third set,[5] the extracted protein seems quite uniform in clones 2470, 2445, and 2555, and somewhat higher in cells of clone 2472. These cells appear to be larger (*4*).

The enzyme activities evidenced by the media are shown in table 3. The growth medium itself was inactive. However, the medium in which

TABLE 3.—Enzyme activities of media

Growth medium		ΔOD/ml/minutes		
		G–6–PD	PGD	No substrate
Fresh from clones				
	2445	0. 006		
	2470	0. 005	0. 002	0
	2555	0. 006		
	2472	0. 005	0. 0024	
Sucrose suspending medium from clones				
	2445	0. 008		
	2470	0. 008		
	2555	0. 014		
	2472	0. 002	0. 007	0

the cells were scraped from the glass and removed from the flasks showed some enzyme activity when 0.5 ml samples were tested after the cells had been centrifuged. This low activity probably represents some breakage of cells during the procedures. The level of activity was approximately the same for all media tested.

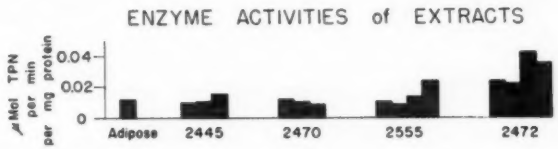
Some of this medium, usually 2 or 3 ml, was carried over with the cells into the sucrose suspension (6–7 ml). This suspending medium also

[5] The values for the nuclear count of the third set of cells is probably low, for the samples were sent through the mail in hot weather.

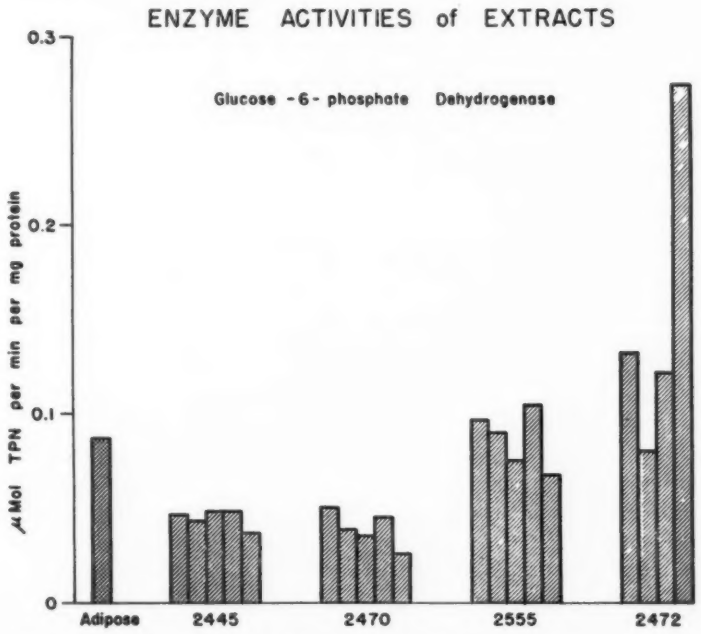
evidenced such low activity (table 3) that it was not applied as a correction for the enzyme activities of the whole cells in suspension.

The specific activities of the enzymes, hexokinase, glucose-6-phosphate dehydrogenase, and phosphogluconate dehydrogenase in extracts of cells of the 4 clones and of normal C3H mouse tissue of the type from which these cells originated, are plotted in text-figures 2, 3, and 4, respectively.

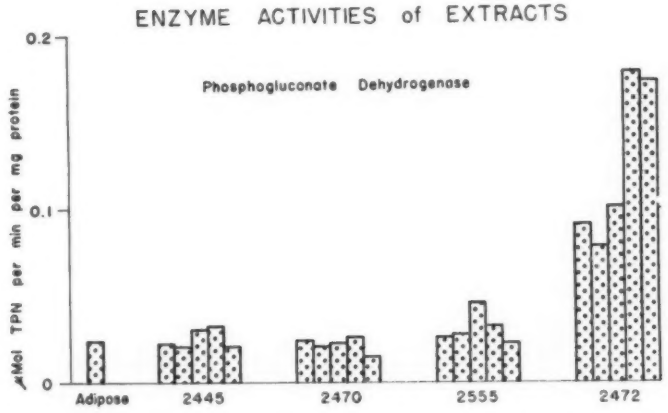
The pattern of enzyme activities of cells of the two clones 2470 and 2445 is almost identical. Cells of the third clone from the low line, clone 2555, a hypertriploid clone that arose from a cell which divided and then fused, have nearly twice the hexokinase and G-6-PD activities but the same phosphogluconate activity as cells of clones 2470 and 2445. The mean of cells of clone 2472 from the high line, as compared with



TEXT-FIGURE 2.—Hexokinase activities of extracts. Each bar represents the mean of two or more determinations of hexokinase activity by the extract of one set of cultures of the clone indicated. The method was as described in the text, and the optical density change was divided by 6.22 to give rate in terms of μmoles of TPN reduced per minute per mg of protein.



TEXT-FIGURE 3.—Glucose-6-phosphate dehydrogenase as in text-figure 2.



TEXT-FIGURE 4.—Phosphogluconate dehydrogenase as in text-figure 2.

clones 2470 and 2445 from the low line, are 3 times higher for hexokinase, 4 times higher for G-6-PD, and 5 times higher for PGD (table 4). There is no overlap between the high PGD values of clone 2472 and the other clones. The hexokinase and PGD of the adipose tissue are similar to those of clones 2470 and 2445. The G-6-PD value was in the range of clones 2555 and 2472.

TABLE 4.—Comparison of means of enzyme activities of various clones

		Means of 2445 and 2470	SD	Mean of 2555 / Mean of 2445 and 2470	Mean of 2472 / Mean of 2445 and 2470
Whole cells μmole TPN 10^9 cells/ minutes	G-6-PD	1. 17	±0. 58	1. 2	4. 0
	PGD	0. 61	±0. 24	0. 95	4. 9
Extracts μmole TPN/ 10^9 cells/ minutes	Hexokinase	1. 5	±0. 74	1. 7	4. 6
	G-6-PD	6. 26	±1. 6	2. 2	6. 0
	PGD	3. 7	±1. 0	1. 2	8. 7
Extracts μmole TPN/ g protein/ minutes	Hexokinase	11. 4	±2. 0	1. 4	2. 7
	G-6-PD	45. 5	±7. 7	2. 1	3. 7
	PGD	23. 6	±5. 0	1. 1	5. 0

The same enzymes have been calculated in terms of activity per 10^9 cells in table 5. The difference between the clone from the high line and clones from the low line is more than eightfold for phosphogluconate dehydrogenase.

The enzyme activities evidenced by the whole cells, when the phosphorylated substrates and TPN were in the suspending medium, ranged from 8 to 35 percent of the activities of the cell-free extracts when calculated per cell. The values are in table 6. Hexokinase can be determined

TABLE 5.—Enzyme activities of cell-free extracts

Clone No.	Set	ΔOD/ml/minutes			ΔOD/10^9 cells/minutes		
		Hexokinase	G-6-PD	PGD	Hexokinase	G-6-PD	PGD
2445	I		2. 50	1. 28			
	II		2. 58	1. 40		42	22
	III	0. 30	1. 95	1. 15	7. 3	47	28
	IV	0. 64	3. 26	2. 2	7. 8	45	30
	V	1. 00	2. 47	1. 4	9. 1	22	13
2470	I		2. 80	1. 35			
	II	0. 60	2. 42	1. 33	12. 5	48	27
	III		1. 50	1. 00			
	IV	0. 54	2. 40	1. 46	11. 0	41	25
	V	0. 50	1. 60	0. 90	7. 8	25	14
2555	I		5. 55	1. 45			
	II	0. 66	5. 30	1. 55	11. 0	87	24
	III	0. 30	3. 00	0. 90			
	IV	0. 75	5. 14	1. 50	18. 0	115	33
	V	1. 24	3. 67	1. 30	18. 8	56	20
2472	I		6. 00	3. 62			
	II	1. 00	4. 20	4. 20	36	155	150
	III	0. 70	4. 07	3. 27			
	IV	1. 20	9. 60	6. 15	40	309	200
	V	0. 78		4. 05	41		236

with whole cells from tissue culture (*18*) but was not attempted in these experiments. The relation of activities among the 4 clones for the whole cells is very similar to the pattern of specific activities, except that the cells of clone 2555 differ less from the other clones from the low line. There is no overlap in values for cells of clone 2472, as compared with cells of the other 3 clones.

The ratios of the means of G-6-PD to PGD were 2.4, 2.0, 2.6, and 1.6 for whole cells of clones 2445, 2470, 2555, and 2472, respectively. The

TABLE 6.—Enzyme activities of whole cells

Clone No.	Set	ΔOD/ml/minutes			ΔOD/10^9 cells/minutes	
		G-6-PD	PGD	No substrate	G-6-PD	PGD
2445	I	0. 10	0. 04		6. 4	2. 6
	II	0. 22	0. 06		14. 6	4. 0
	III	0. 07	0. 04		4. 7	2. 7
	IV	0. 187	0. 128	0. 077	4. 7	4. 2
2470	I	0. 15	0. 07	0	4. 8	2. 2
	II	0. 12	0. 08		10. 8	7. 2
	III	0. 10				
	IV	0. 08	0. 07		4. 2	3. 7
2555	I	0. 25			10. 3	
	II	0. 10	0. 05		8. 1	4. 1
	III	0. 26	0. 07			
	IV	0. 17	0. 08	0. 037	7. 0	3. 3
2472	I	0. 50	0. 19	0	37	14
	II	0. 16–0. 24	0. 22		20–30	28
	III	0. 114	0. 09			
	IV	0. 23	0. 155	0	23	15. 5

567420—60——16

same ratios for the extracts were 1.74, 1.77, 3.37, and 1.37, respectively, in the same order.

DISCUSSION

Although these enzymes dealing with the oxidative catabolism of glucose are widely distributed in microorganisms, plants, and different mammalian tissues and tumors (*19*, *20*), their activities in mammalian cells during culture *in vitro* have not been extensively investigated. Perske, Parks, and Walker (*21*) examined a cultured cell line from human liver (Chang's liver strain) for certain enzymes reputed to characterize liver parenchymal cells *in vivo*. It was significant that the only enzymatic activity detected in the cultured cells was that of G-6-PD, which in normal rat liver is the least active of the enzymes considered. The extractable activity of G-6-PD remained substantially unchanged after the cells were carried for 4 subcultures in a medium containing fructose rather than glucose as the major carbohydrate source.

Lieberman and Ove (*22*) compared several enzyme activity levels in rabbit kidney cortex and primary 7- and 22-day-old cultures derived from this tissue. Many activities in the cultured cells were markedly different from those in the original tissue and showed variations in the direction of levels encountered in 4 long-term human cell strains also studied. A conspicuous rise in G-6-PD activity was noted in the cultured cells as compared with the original tissue.

Burlington (*23*) found the combined activities of G-6-PD and PGD 40 percent higher in primary (4- to 8-day-old) cultures of kidney cortex cells from the cat than in the original tissue. All other enzymes studied showed severely reduced levels of activity in culture. The author suggested that the "hexose monophosphate shunt" for glucose oxidation might be operating at greater than normal rate in cultures, possibly supplying ribose units for nucleotide synthesis in the dividing cells.

However, in both of these studies comparing primary cultures with original tissues, the activity of any enzyme assayed represented an average value for a mixed-cell population. As pointed out by Burlington, the pattern emerging in culture could result from either cell selection or actual change in enzyme activity induced by the nutritional and physical conditions *in vitro*.

An advantage of the present system is that all four clones studied originated in culture from the same cell, the progeny of which were grown under similar culture conditions and in the same lots of medium. Thus, any differences in enzyme activity emerging in culture must have resulted from actual change in the cells during their growth *in vitro*.

In spite of the years of cultivation *in vitro* and the selection, both during growth in culture and by cloning, the activities of the enzymes studied were, in general, similar in the tissue-culture cells and the tissue of origin. Apparently growth in culture did not select for these particular enzymes, nor for their deletion.

It is also clear from this series of analyses that heritable differences in enzyme activities did emerge in culture from one cell, and that the difference between the high-line clone and the 3 low-line clones was greater than that between the low-line clones. The degree of divergence is possibly related to the length of time the cells were carried as distinct and separate cell populations. The high line was separated from the low line for 5 years prior to initiation of the clones, whereas the clones were carried for only 2 years before analysis.

In general, all three enzymes showed the highest activity in cells of clone 2472 from the high line. It will be recalled that cells of this clone also grew most rapidly into palpable tumors in mice (though growing at approximately the same rate as low-line cells in culture), showed a higher rate of anaerobic glycolysis *in vitro*, and initiated a rapid vascular response in mouse tissue seldom or never observed with implants of low-line cells. The greater capability of the high-line cells to produce a vascular response in host tissues may result from some substance produced by the cells, either a metabolite qualitatively or quantitatively different from any formed by low-line cells or possibly a polypeptide. The work of Menkin (*24*) indicates that polypeptides and nucleopeptides produced during an inflammatory reaction have such activity. From all these considerations, it would appear that cells of the high-line clone may be more metabolically active cells and that their high tumor-producing capacity may be associated with the increased activity of certain of these enzymes, possibly PGD.

When enzyme activities in the three low-line clones were compared, both hexokinase and G-6-PD were higher in the hypertriploid clone 2555, and activities were within the lower range of the high-line clone 2472. Clone 2555 had the least capacity of the three low-line clones to produce tumors, which developed only if the host mice were given a prior dose of irradiation to damage immune mechanisms (*4*). It seems possible that the hypertriploid condition of the chromosomes may have so altered the genetic constitution of these cells as to make them immunologically incompatible with the C3H mouse. If so, attempts to correlate patterns of enzyme activity with tumor-producing capacity of cells would be difficult and complicated by factors influencing transplantability.

The cause of the observed heritable difference in enzyme activities is not clear. Evidence from genetic studies indicates that genes in some manner control the potentiality for enzyme formation. In microorganisms, plants, and higher animals including mammals, enzymatic consequences of a changed genotype have been identified with great precision (*25–28*). That these clones have undergone genetic change is evidenced by the increase in chromosome numbers and the morphologic change in size, shape, and centromere location in specific chromosomes (*5*). Since these clones originated from a highly inbred, presumably homozygous mouse, the effect of increased chromosome number would primarily affect gene dosage, but not qualitatively alter the genome. The morphologic alterations in chromosomes suggest that fragmentation, inversions,

or translocations may have occurred which introduced position effects. These two effects might conceivably account for differences in enzyme activities. Until mouse chromosome numbers and morphology can be better stabilized in culture, this possiblity cannot be disregarded. A second possibility is that gene mutation and selection account for emergence of the different cell types.

A third possibility is that some process similar to embryological differentiation occurred in culture, but it was a less orderly, more aberrant differentiation. During cell differentiation, a progressive change in the quantitative distribution of enzymes occurs without apparent change in chromosome structure. Each adult tissue is thus characterized by a unique pattern of enzyme content (*29*, *30*). The nature of this process is unknown and may be associated with local environmental conditions and cytoplasmic segregation of substrates and interactants (*27*, *31*).

In addition to what appear to be heritable differences in enzyme levels between the clones studied here, considerable variability is also noted. All 3 enzymes showed a greater degree of variation in the high-line clone 2472 than in the other 3 clones. The activities were higher in the last 2 analyses done in October than in those in the spring.

Studies of the activity of these enzymes in extracts of *Escherichia coli* (*32–34*) have shown a pattern of G-6 PD and hexokinase variability induced in response to certain changes in the growth medium, while the specific activity of the PGD was much more constant. The response of G-6-PD and hexokinase to activators and inhibitors *in vitro* is also much greater than PGD. The outstanding exception to this pattern is that a high TPNH/TPN ratio is inhibitory to PGD and not to G-6-P (*35*) *in vitro*. It is possible that the maintenance of a high ratio is involved in the control of PGD activity and perhaps also to adaptation *in vivo*. Glock and McLean have found that levels of total TPN are low in tumors, and the TPNH/TPN ratios, calculated from their figures, are lower than in normal tissues (*36*). All DPNH/DPN ratios were less than 1, and differences in ratios between normal and tumor tissue were not marked. It might be speculated that something in the medium or the gas phase might have affected this ratio and allowed an adaptive increase of PGD. Increased PGD activity might have provided more ribose phosphate for synthetic processes and led to greater capacity for growth.

Although the cells studied here were grown under similar culture conditions in the same lots of culture fluid, possible crowding effects could not be prevented or accurately controlled in these stationary cultures. As emphasized by Loomis (*37*), crowding respiring cells together can produce localized gradients of decreased 0_2 tension, increased CO_2, decreased *p*H, increased NH_3 tension, decreased concentration of nutrients and vitamins, and increased temperature. Loomis found that sexual differentiations of *Hydra littoralis* depended on CO_2 tension.

Yamada (*38*) noted changes in morphology from epithelial to fibroblast-like cells of a culture of HeLa strain cells after prolonged culture, 30 or more days without change of medium or adjustment of *p*H. The culture

appeared to degenerate, the cells fell off the glass, and later new colonies of cells with elongated shape like fibroblasts formed on the surface. This morphology was stable on subsequent culture and passage. Yamada considered this phenomenon to be due to spontaneous mutation and selection. Chèvremont and Chèvremont-Comhaire (*39*) have studied a transformation of skeletal muscle cells or subcutaneous connective tissue in primary culture from fibroblast form to histiocyte or macrophage. The agent responsible was found in the cultures and proved to be choline. Added choline had the same effect. After several passages the cells were resistant to the action of choline.

Wilde (*40*) has studied differentiation of embryonic cells of the neural crest in tissue culture. Neuroepithelial cells differentiated into pigment cells under the influence of phenylalanine, supplied to the medium either exogenously or by cells of the archenteron roof mesoderm. At slightly higher levels of phenylalanine concentration, even cells from the ventral ectoderm of the gastrula could differentiate to pigment cells. Using various analogues of phenylalanine, Wilde has been able to show different types of inhibition and interaction in patterns of differentiation. These changes are morphological and functional and due to specific chemical effects, not to mutation. Another similar effect is the relationship of vitamin A to the morphology of epithelial cells (*41*).

Wilde (*42*) also reports that he and others have found that if, after disaggregation by trypsin, Versene, or by Ca^{++} free solution, embryonic cells are washed and cultured, no differentiation can be noted in dilute suspensions. In a denser suspension, if the culture fluid is not changed, differentiation may occur after many hours in some cells. Substances released into the medium during disaggregation caused some degree of differentiation, if added back to the suspension. Wilde suggests that the frequent changes of medium during tissue culture prevent the accumulation of substances in the medium which might cause differentiation, and thus the cells are maintained in the undifferentiated state. On this basis Yamada's results might be explained as changes due to accumulation of such substances during prolonged culture rather than to mutation.

With better control of culture conditions and with stabilization of chromosomes, further studies on these enzyme activities accompanying deliberate changes in variables of culture should be illuminating.

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Serum-Protein Changes in Malignant Disease. II. The Chronic Leukemias, Hodgkin's Disease, and Malignant Melanoma [1]

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SUMMARY

Four malignant neoplasms were investigated and each showed differing changes in the serum proteins. Uncomplicated chronic lymphocytic leukemia was characterized by hypogammaglobulinemia, chronic myelocytic leukemia by hypergammaglobulinemia, Hodgkin's disease by increased α-globulins and decreased albumin, and malignant melanoma by lowered serum-albumin levels. The changes with each malignant disease depend, in part at least, on the stage of disease activity. Hodgkin's disease strikingly displayed this fact where disease remission was associated with complete return of the serum-protein pattern to normal. Serum-albumin levels remained normal in most patients with uncomplicated chronic leukemia, even in the late stage of disease; the β-globulin level was little altered, even in the presence of marked changes in other serum proteins, and no characteristic pattern of serum-protein response could be found for all malignancy *per se*. Frequent complications of many malignant processes, such as infection, fever of undetermined etiology, or liver disease, produced further protein changes that were a reflection of the particular complication rather than of the primary type of neoplastic disease. Nitrogen mustard, tris(1-aziridinyl) phosphine sulfide (thio-TEPA), and adrenocortical steroids could not be shown to influence serum-protein levels directly.—J. Nat. Cancer Inst. 1381–1390, 1960.

THE SERUM-PROTEIN abnormalities most commonly found in association with malignant neoplastic disease are variations in the levels of normally present proteins. The resultant serum-protein electrophoretic pattern, although not diagnostic, may be altered in a consistent and characteristic fashion for a particular neoplasm. In a previous study (*1*) we found that the two common forms of acute leukemia, myeloblastic and lymphoblastic, were associated with differing serum-protein changes. Myeloblastic leukemia was characterized by an elevation of γ-globulin, while lymphoblastic leukemia was characterized by an elevation of the α-2 globulin. We were able to distinguish changes apparently induced

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in the serum proteins by the leukemic process from effects of the more obvious complications, such as fever, infection, and liver disease.

This study is a similar investigation of the serum-protein changes associated with the chronic leukemias and Hodgkin's disease. The effects of the severity of the illness, the superimposed changes of complications, and the possible effect of chemotherapeutic agents were assessed. The effect of chemotherapeutic agents on serum proteins has not been well assessed in man, and to avoid confusing the direct effect of such therapy with indirect results of improvement in the disease, patients with malignant melanoma were included. These patients and those with Hodgkin's disease received the same form of therapy, but improvement was rare in the former.

Each disease was associated with differing serum-protein changes. The most marked changes, hypogammaglobulinemia in chronic lymphocytic leukemia, hypergammaglobulinemia in chronic myelocytic leukemia, and increased α-2 globulin in Hodgkin's disease, have been noted by others (*2–12*). The serum-protein alterations of Hodgkin's disease reflect the severity of the illness, but this relationship is less clear in the other diseases. There was little evidence of an effect on the serum proteins of cytotoxic drugs or adrenocorticosteroids. The complications were associated with further changes in the serum proteins which were characteristic of the complication rather than the malignant disease.

MATERIALS AND METHODS

The procedures for collecting serum samples, performing protein and quantitative paper electrophoretic analyses, separately evaluating clinical data including complications, and making statistical analyses have been described (*1*). One hundred and eighty-five samples were obtained from 32 patients with chronic lymphocytic leukemia, 4 of whom had a single serum-protein determination. Sixty-five samples were obtained from 15 patients with chronic myelocytic leukemia, 6 of whom had single determinations. A total of 65 samples was obtained from 15 patients with Hodgkin's disease, and 76 samples were obtained from 19 patients with malignant melanoma. All patients with Hodgkin's disease and malignant melanoma had at least 2 samples analyzed. The criteria for clinical evaluation are noted.

Hodgkin's disease.—The diagnosis in each case was established by lymph-node biopsy. The severity of illness was established by the use of the size of lymph nodes and tumor infiltration as the primary criterion, though hemoglobin values, symptoms, and such complications as bronchial constriction by tumor were considered. A "0" to "4+" grading system was used. No attempt was made to equate directly the severity of one patient's illness to that of another on the basis of the findings. Rather, in each case the severity of the patient's illness at one time was graded

against the severity of illness at another time. A "0" disease-activity designation meant a patient had no physical evidence of disease and was asymptomatic. In contrast, "4+" activity meant that he was so ill that death was considered imminent. The "1+," "2+," and "3+" were between these two extremes.

Chronic lymphocytic leukemia.—Diagnosis was established by bone marrow and/or peripheral blood examination. All patients had an elevated white blood cell count with lymphocytosis at some time during the period of observation. Disease activity was graded on the following bases: "0" disease activity = asymptomatic, no enlargement of lymph nodes, spleen, or liver, over 13 g hemoglobin/100 ml whole blood, over 150,000 platelets per mm^3, over 2,000 mature granulocytes per mm^3; "1+" disease = "0" plus lymphadenopathy, lymph nodes no greater than 2 cm in diameter, and/or spleen and liver less than 2 cm below the costal margin and/or mild symptoms of fatigue; "2+" disease = more evidence of disease than in "1+," but hemoglobin of at least 10 g percent, platelets at least 100,000 per mm^3, patient ambulatory and fairly comfortable; "3+" disease = patient still ambulatory though severely symptomatic; "4+" disease = patient considered near death and generally confined to bed.

Chronic myelocytic leukemia.—Diagnosis was established by marrow and/or peripheral blood examination and disease activity was graded: "0" activity = the same as chronic lymphocytic leukemia and essentially no myeloblasts in peripheral blood; "1+" disease = the same as chronic lymphocytic leukemia and essentially no myeloblasts in the peripheral blood; "2+" disease = same as chronic lymphocytic leukemia with no more than 10 percent myeloblasts in the peripheral blood; "3+" and "4+" diseases = same as chronic lymphocytic leukemia.

Malignant melanoma.—All patients had inoperable, metastatic disease proved by biopsy. No gradation of disease activity was applied. No patient demonstrated improvement, and the majority of patients with sequential serum-protein samples were clinically worse with each succeeding sample.

Antitumor therapy.—Serum-protein values from patients receiving therapy with the various drugs were compared to values from patients (often the same patient) with comparable disease activity receiving no therapy. Most patients with Hodgkin's disease and malignant melanoma received treatment with either tris(1-aziridinyl)phosphine sulfide (thio-TEPA) or nitrogen mustard, as described by Zubrod and associates (*13*). In each instance a 12-week course of therapy was given with various modifications for the degree of toxicity induced. The standard course of nitrogen mustard was 0.1 mg/kg daily for 4 days and, after a 2-week waiting period, the same dose at weekly intervals. Thio-TEPA was given on the same schedule at 0.2 mg/kg. For the treatment of chronic myelocytic leukemia, Myleran or colchicine derivatives were used in varying dosage schedules, and maintenance therapy was usually employed. Most patients with chronic lymphocytic leukemia were treated

with 1 mg/kg/day of prednisone for 4 weeks, 0.5 mg for 4 weeks, and 0.25 mg for 4 more weeks. In all diseases, a variety of antitumor agents was used other than those considered, but the number of analyses during other forms of therapy was too small for meaningful study of the possible effects of the drugs.

Most patients with Hodgkin's disease, lymphocytic leukemia, or myelocytic leukemia were benefited by the therapy; as previously mentioned, no improvement was noted in those with malignant melanoma.

RESULTS

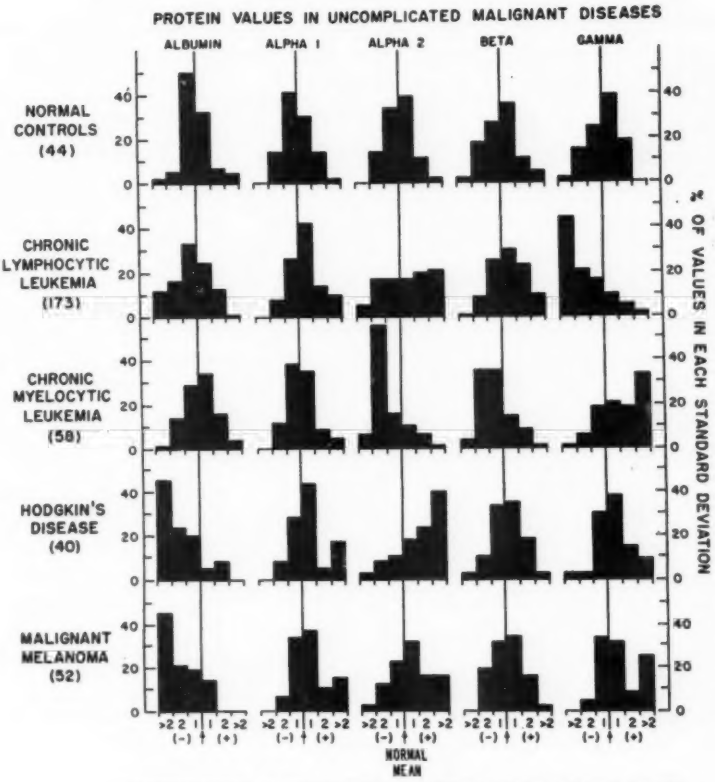
Each of the diseases investigated produced significant and differing serum-protein changes. Median values and the range for each component obtained by analysis of uncomplicated serum samples are assembled in table 1. The distribution of analytic values is graphically presented in text-figure 1.

TABLE 1.—Serum-protein values in uncomplicated disease

		Albumin	α-1	α-2	β	γ
		(Values in grams percent)				
Normal	Mean	4. 16	0. 30	0. 68	0. 77	1. 17
	Range (± 2SD)	3. 52–4. 80	0. 20–0. 40	0. 48–0. 88	0. 57–0. 97	0. 77–1. 57
		(Values in % of normal mean)				
Chronic lymphocytic leukemia	Median	95	110	105	105	60
	Range	25–130	55–215	45–240	60–165	15–170
Chronic myelocytic leukemia	Median	100	100	80	90	125
	Range	60–150	60–160	35–145	45–185	55–225
Hodgkin's disease	Median	75	115	130	100	110
	Range	25–120	65–195	60–235	55–140	30–260
Malignant melanoma	Median	75	110	115	100	105
	Range	40–110	70–195	60–215	60–155	50–210

Chronic lymphocytic leukemia was characterized by hypogammaglobulinemia. The median γ-globulin level was 60 percent of normal ($P = <0.0001$) and 44 percent of values were more than 2 standard deviations (SD) below the normal mean. In contrast, the serum-albumin level usually was normal, though 21 of the 173 values were more than 2 SD below the normal mean ($P = <0.01$). The α- and β-globulin groups were variable, but the usual finding was a slight increase above the normal mean of these 3 globulin families.

Chronic myelocytic leukemia differed from chronic lymphocytic leukemia most strikingly in the high frequency of hypergammaglobulinemia (33% of values more than 2 SD above the normal mean). The albumin



TEXT-FIGURE 1.—The serum-protein observations are grouped in relation to the normal median value and the normal standard-deviation value for each component. These analyses include all the serum samples obtained during uncomplicated periods of the diseases, regardless of severity of disease.

was normally distributed, as were the α-1 globulins. Although marked decrease in α-2 and β-globulins was unusual, these components tended to be decreased, and three fourths of these globulin values in chronic myelocytic leukemia fell below the normal mean ($P = < 0.01$).

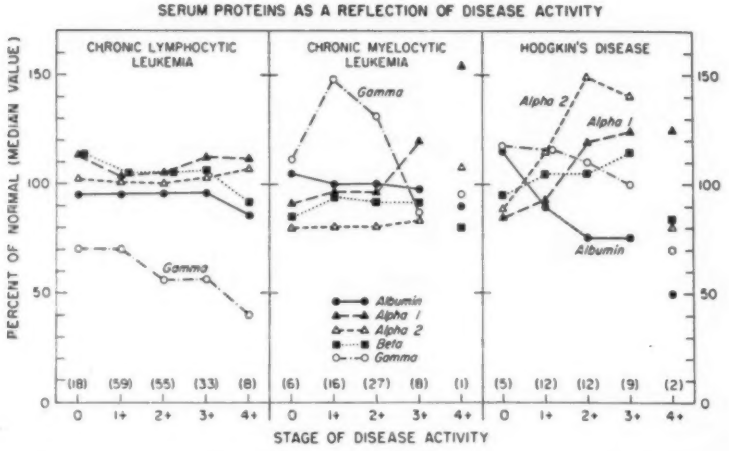
Hodgkin's disease characteristically showed elevated α-2 globulins (40% of values were more than 2 SD above the normal mean, $P = < 0.001$) and decreased albumin (46% of values under 2 SD below the normal mean, $P = < 0.0001$). The median values for α-1, β-, or γ-globulins were not significantly different from the normal mean, but an appreciable number of values of α-1 and γ-globulins was more than 2 SD above the normal mean ($P = < 0.05$).

Malignant melanoma was usually associated with decreased serum-albumin levels (45% of values more than 2 SD below the normal mean).

The median value for the other protein components did not differ significantly from the normal mean, but α- and γ-globulin levels above the normal range were frequent ($P = < 0.05$).

Relation of Serum-Protein Changes to Activity of Neoplastic Disease

A close correlation between the severity of disease and the serum-protein changes was seen in Hodgkin's disease (text-fig. 2). With increasingly severe disease, the albumin fell progressively, and both the α-2 and α-1 globulin components rose. With subsidence of disease activity these serum-protein changes regressed and the albumin, α-2 and α-1 globulin levels returned to normal. During the course of uncomplicated Hodgkin's disease, β- and γ-globulin levels did not vary significantly.



TEXT-FIGURE 2.—The median serum-protein values at various stages of uncomplicated disease activity are recorded. The number of measurements is noted in parentheses above each stage of disease activity.

In the chronic leukemias the only changes were in the γ-globulins. Albumin, α- and β-globulin values did not appreciably vary with progression or regression of these diseases. The γ-globulins were usually normal in chronic myelocytic leukemia during complete remission and rose as the disease became active. In very sick patients the level may fall to normal or, occasionally, low levels. In chronic lymphocytic leukemia, the γ-globulins were frequently decreased in patients with early or little disease activity. These already lowered γ-globulin levels showed a progressive fall with increasing severity of the lymphocytic leukemia. A slight rise, without return to normal, was noted in some instances of disease improvement.

Effect of Antitumor Therapy on Serum Proteins

The effect of nitrogen mustard and thio-TEPA on the serum proteins was evaluated in patients with Hodgkin's disease and malignant melanoma (table 2). Twenty-three patients who received these alkylating agents without effect on their disease showed essentially no change in serum electrophoretic components. Similarly, the median values obtained from patients with chronic lymphocytic leukemia who were being treated with prednisone and from patients with chronic myelocytic leukemia who were being treated with Myleran failed to differ from values obtained in untreated patients with comparable disease activity. None of the serum-protein changes noted during disease could be attributed to these therapeutic agents.

Fever, Bacterial Infection, or Liver Disease

When one of these complications was superimposed on the malignant disease, further changes were evident in the serum proteins. Determinations were available during 24 episodes of bacterial infection (12 in lymphocytic leukemia, 2 in myelocytic leukemia, 4 in Hodgkin's disease, and 6 in malignant melanoma). In 19 of the 24 instances, the albumin was below the median value for similar patients with comparable disease activity ($P = < 0.01$). The α-1 globulin was increased in 19 of the 24 infections ($P = < 0.01$). Fever of undetermined etiology (5 episodes in myelocytic leukemia, 14 in Hodgkin's disease, and 10 in melanoma) was associated with an increase in the α-1 globulins in 21 of the 29 episodes ($P = < 0.05$). Liver disease, presumably secondary to malignant invasion, complicated 20 determinations (7 in Hodgkin's disease and 13 in melanoma) and resulted in further decrease of the albumin in 18 of the 20 values ($P = < 0.01$). Other electrophoretic groups were not significantly altered by these common complications of malignant disease. Similar protein alterations were associated with these complications in acute leukemia (*1*), which indicates that the protein change is a reflection of the complication rather than of the type of underlying neoplastic disease.

DISCUSSION

The differing serum-protein changes found in each disease were clearly revealed by analyzing only serums obtained during uncomplicated disease and by classifying the patients as to stage of disease activity. Common complications of malignant disease such as infection or hepatic dysfunction produce serum-protein changes that are characteristic of the complication and not of the underlying malignant disease.

The α-2 and α-1 globulin increases of active Hodgkin's disease are among the most marked in disease. A detailed study of the α-globulin groups

TABLE 2.—Serum-protein changes during HN2 or thio-TEPA administration

Disease	Albumin		α-1		α-2		β		γ	
	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease
Improvement:										
Hodgkin's disease	*10*	4	4	*10*	5	*9*	6	8	6	8
No improvement or progression of:										
Hodgkin's disease	2	4	4	2	3	3	3	3	3	3
Melanoma	4	13	8	9	8	9	9	8	5	12

in uncomplicated Hodgkin's disease has been made by Goulian and Fahey (*14*) employing anion-exchange chromatography in conjunction with electrophoretic fractionation procedures. Seven α-globulin components were found in normal serums, and 2 of the 7 components, one an α-1 globulin and the other an α-2 globulin, accounted for the bulk of the increase seen by electrophoresis in active Hodgkin's disease. One of the α-2 globulin components had increased to 300 and 450 percent of the normal level, which indicates that additional information can be obtained by the study of individual proteins within the serum electrophoretic groups.

The α-1 globulin level in chronic myelocytic leukemia was of particular interest because of the 2- to 30-fold increase that occurs in the α-1 vitamin B_{12}-binding protein. This change is usually expressed in terms of the increased serum B_{12} level, but the serum B_{12} level and serum vitamin B_{12}-binding capacity, *i.e.*, the level of B_{12}-binding protein, increase together in chronic myelocytic leukemia (*15*, *16*). The B_{12}-binding protein comprizes only about 0.003 percent of the normal total α-1 globulin group (*17*). Even a 30-fold increase of the B_{12}-binding protein, as had occurred in several of these serums, would have little impact on the electrophoretic measurement, and in this study the electrophoretic α-1 globulin group was not increased in uncomplicated chronic myelogenous leukemia.

The hypogammaglobulinemia found in chronic lymphocytic leukemia is of clinical as well as theoretical interest. Study of antibody response to antigenic stimulation, γ-globulin levels, and frequency of infection in the patients with chronic lymphocytic leukemia indicated that there was a correlation between susceptibility to infection, impaired antibody production, and the presence of hypogammaglobulinemia (*12*). A high frequency of hypogammaglobulinemia has been noted previously in chronic lymphocytic leukemia and a general correlation with the occurrence of infection established (*10*, *11*). Several of the patients in the present study with hypogammaglobulinemia had never received antileukemic therapy of any kind, which indicates that this altered function was a property of the disease and not due to chemical or radiation therapy. The reasons for the low γ-globulin in lymphocytic leukemia and the elevated γ-globulin in myelocytic leukemia are obscure.

The serum-protein changes were notably different in the diseases we studied. In various carcinomatous diseases, the changes appear to be more uniform (*18*). The specific serum-protein changes may be due to direct effects of the type of neoplastic cell and to the extent of neoplasm encroachment on normal host metabolism. This view is supported by the data in text-figure 2 and by other observations of a general correlation between neoplastic disease activity and serum-protein change (*2–12*, *18*), but additional factors may influence the serum-protein changes, for such correlation is poor in the acute leukemias (*1*). Studies such as the present one indicate some specificity in the changes produced by different neoplasms and lend hope that additional studies, particularly of individual protein components, will increase the understanding of the significance of

serum-protein changes and the clinical usefulness of detecting serum-protein alterations in malignant disease.

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Importance of the Infecting Dose on Growth Patterns of Rous Sarcoma Virus (RSV) in Chick Brain[1, 2]

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SUMMARY

The growth curves of Rous sarcoma virus (RSV) in chick brain paralleled those of many nononcogenic animal viruses only when large doses were used. First, virus disappeared very rapidly from the brain after intracerebral inoculation. This was followed by a stationary phase of varying duration, during which the infective virus content of the brain remained low. Finally, the relative potencies of brain tissue increased logarithmically and reached their peak in moribund chicks. Growth curves were obtained for each of six different doses of RSV. It was found that the length of the stationary phase, the time to double-extractable potency, and the maximum potency attained in brain tissue were directly related to the amount of virus contained in the infecting inoculums. It is suggested that the dependence of the pathogenesis of disease on the amount of RSV contained in the infecting dose represents a basic difference between at least one tumor virus and the nononcogenic viruses.—J. Nat. Cancer Inst. 25: 1391–1404, 1960.

PREVIOUS REPORTS (*1–3*) have shown that the brain of newly hatched chicks offered a useful host-virus system for biological studies on the Rous sarcoma virus (RSV). The virus propagated in the brain of young chicks (*1*) and killed them according to amount of dose (*1*, *2*). No gross lesions were observed in the brain, but a pathognomonic hemorrhagic area was almost invariably found in the meninges (*1*). Histologically, Rous sarcoma cells, similar to those described by Vázquez-López (*4*) and by Duran-Reynals (*5*), were seen beneath the meninges and recently have been observed invading the brain parenchyma (*6*). Further studies showed that the time-to-death response of chicks inoculated intracerebrally with serial decimal dilutions of RSV was consistently linear and that this system could be employed as another method for bioassay of infectivity (*1*, *2*). Subsequently, it was shown that: (*a*) Prior intracerebral inoculation of viable or heat-inactivated influenza B virus exerted a sparing effect on mortality in chicks resulting from the intracerebral

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inoculation of RSV (*1*). This sparing effect resembled interference between viruses rather than an oncolytic action, inasmuch as heat-inactivated virus was equally as effective as viable influenza virus. (*b*) The virus was propagated serially for 35 passages in chick brain and the potency of the 35th passage was substantially higher (1.5 logs) than that of brain tissue from the first passage (*3*). It was also reported that the incidence and severity of hemorrhagic disease, originally described by Duran-Reynals (*7*), increased with successive passages (*3*).

More recently it was reported that the intracerebral host-virus system was adaptable to studies on the viral growth curve and also to studies on the relationship of infecting dose to yield of virus in chick brain (*2*). The latter study confirmed the work of Bryan, Calnan, and Moloney (*8*), who showed that the amount of infective virus demonstrable in individual chicken sarcomas was directly related to the infecting dose of RSV. This relationship appears to represent a basic difference between host response to at least one oncogenic virus and the response to many nononcogenic viruses as represented by influenza, Newcastle disease, and pneumonia virus of mice (PVM).

The studies described in this report were undertaken to determine the extent to which the pathogenesis of disease in the chick-RSV system was dependent on the infecting dose of virus.

MATERIALS AND METHODS

Sources and preparation of RSV.—Stable, standard RSV was provided by Dr. W. Ray Bryan, of the National Cancer Institute. Such frozen virus was prepared in citrate buffer by differential centrifugation of selected tumor tissues and stored in a dry-ice chest at −60° to −70° C (*9*). In several experiments, 10× concentrated RSV prepared from chick brains of the 40th to 50th serial intracerebral passages initiated with standard virus was used and was prepared as follows: Whole brains, removed aseptically from moribund chicks, were pooled and stored at −65° C in glass-sealed tubes. Immediately before use the brain tissue was thawed, ground in a mortar with sand, and sufficient diluent (*2*) was added to make a final concentration of 10 percent brain tissue by weight. The suspension was then clarified by centrifugation in a Model PR-2 International refrigerated centrifuge at 2500 rpm (1700 × *g*) for 10 minutes. The supernatant fluid was further clarified at 5000 rpm (2178 × *g*) for 30 minutes in a Spinco Model L ultracentrifuge. Lastly, the homogenate was centrifuged at 30,000 rpm (75,000 × *g*) for 30 minutes in the ultracentrifuge. The pelletized material was thoroughly resuspended in one tenth the original volume and immediately inoculated into chicks.

Animals.—Unsexed, White Leghorn chicks were received within 24 hours of hatching and were supplied by a reliable local hatchery. All animals were held in standard-type batteries at incubating temperatures

of 40° C for 2 days prior to inoculation, after which the runts were discarded. All birds were fed dry Turkey Startena (Purina Co.) and water *ad libitum*. Each bird was individually identified with a numbered, metal wing band and also color-coded with dye, according to its experimental or control group, to facilitate rapid identification.

Inoculation of birds.—Chicks were placed under light ether anesthesia and inoculated intracerebrally into the right hemisphere with 0.05 ml of inoculum by means of a ¼-inch, 26-gauge hypodermic needle. All chicks were observed daily for a period of up to 5 weeks, and each chick was examined at time of death for the presence of the pathognomonic lesion in the meninges at the site of inoculation (*1*). Subcutaneous inoculation into the wing web was carried out as follows: Two-tenths ml amounts were inoculated subcutaneously into the wing web with a ¾-inch, 26-gauge hypodermic needle. The needle was inserted through the muscle into the subcutaneous tissue of the left wing web to avoid leakage. The birds were examined daily for 2 to 4 weeks, depending on the potency of the inoculum.

Preparation of brain tissue for bioassay.—The method employed for collection, storage, and extraction of infected brain tissue has been described previously (*2*). Briefly, the method consisted of removing brains aseptically from 3 to 5 birds per day during the 10 to 25 days of the growth-curve experiment. The number of days that brain tissue was collected depended on the infecting dose of virus used for each growth curve (*see* "Results"). Brain tissue was removed only from apparently healthy or moribund chicks and each of the 3 to 5 brains harvested per day was divided in such a manner that 2 comparable pools of tissue were made available for assay. All pools of tissue were stored at −65° C until assayed. On the day of assay, 10 percent extracts were prepared of half of each pool of brain tissue and immediately inoculated into test groups of chicks so that the time between removal of the tissue from the dry-ice chest and inoculation of the last bird did not exceed 2 hours.

Analysis of the data.—Time-to-death and latent-period responses of tumors were analyzed by the graphic probit method of analysis recently illustrated in detail (*10–12*). The application of this method to studies on intracerebral growth patterns has been described (*2*). The metameter most desirable for analyses of these data was the reciprocal of time in days, multiplied by 100 (100/days) to avoid decimal fractional values. The graphic method was chosen because of the presence, in any batch of test chicks, of aberrant, highly resistant animals, whose plotted responses were truncated with respect to the remainder of the linear-response curve used for determining the time to kill 50 percent of the test chicks ($y50$).

For the comparison of groups of chicks which had been inoculated with graded doses of RSV or with virus extracted from brain tissue, the time-to-death or time to produce tumors in 50 percent of the chicks was estimated by the following procedure: The numbers of individuals responding per day for successive day intervals were accumulated, the numbers were converted to percentages and then plotted on probability paper [4]

[4] No. 32,451 (normal ruling), Codex Book Co., Norwood, Mass.

against time expressed as 100/days. The time-frequency relationships were linear in this form, and facilitated graphic analysis. Finally, by an arbitrary assignment of a 100 percent potency to standard RSV (*2*), it was possible to determine the relative potencies of homogenates of brain tissue (*2*).

In several experiments, the potencies of either standard RSV or of RSV from 10 percent extracts were assayed by an improved pock-counting method, with the use of the chorio-allantoic membrane (CAM) of embryonated hens' eggs (*13*). The virus titers obtained by this method are expressed as the log number of pock-forming units (PFU) per gram of tissue and closely parallel virus titers as measured by the intracerebral (*2*, *3*) or subcutaneous (*10*) methods of assay in chicks.

RESULTS

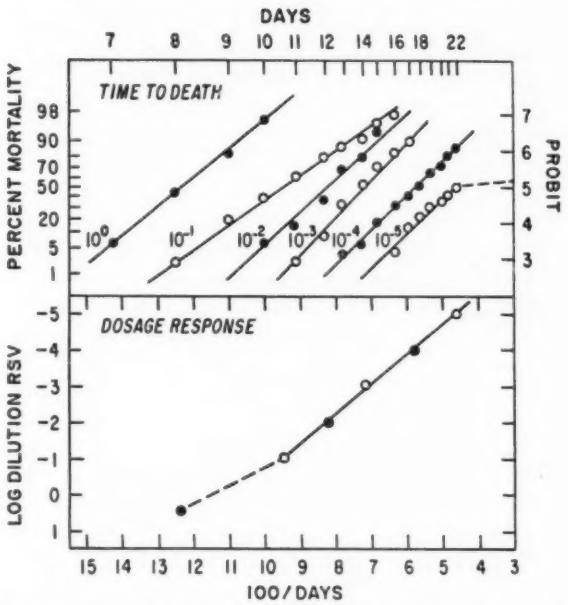
Growth Curves of RSV in Chick Brain

The general experimental design for determining the growth patterns of RSV after intracerebral inoculation was as follows: Five serial decimal dilutions of standard RSV (10^{-1} through 10^{-5} containing approximately 10,000 to 1 ED50, respectively) were prepared and each was inoculated into large numbers of young chicks. The number of birds (100 to 275) inoculated per dilution group was determined from previous experiments which allowed estimation of the average time-to-death for each dilution of RSV. To study growth patterns in chicks inoculated with a preparation of RSV more potent than the standard RSV, a sixth group was similarly inoculated with virus extracted from infected brain tissue of the 35th serial intracerebral passage (*3*). This preparation assayed 1.4 logs higher than standard RSV.

Immediately after inoculation, each of the 6 groups of chicks was divided at random into 2 subgroups. Subgroup A of each group contained 40 to 45 birds that served as the controls. The chickens were observed daily for determining time-to-death. Subgroup B of each group contained the remaining chicks, which were used for the viral growth determinations; 3 to 5 birds were killed daily and their brain tissue was harvested for subsequent assay.

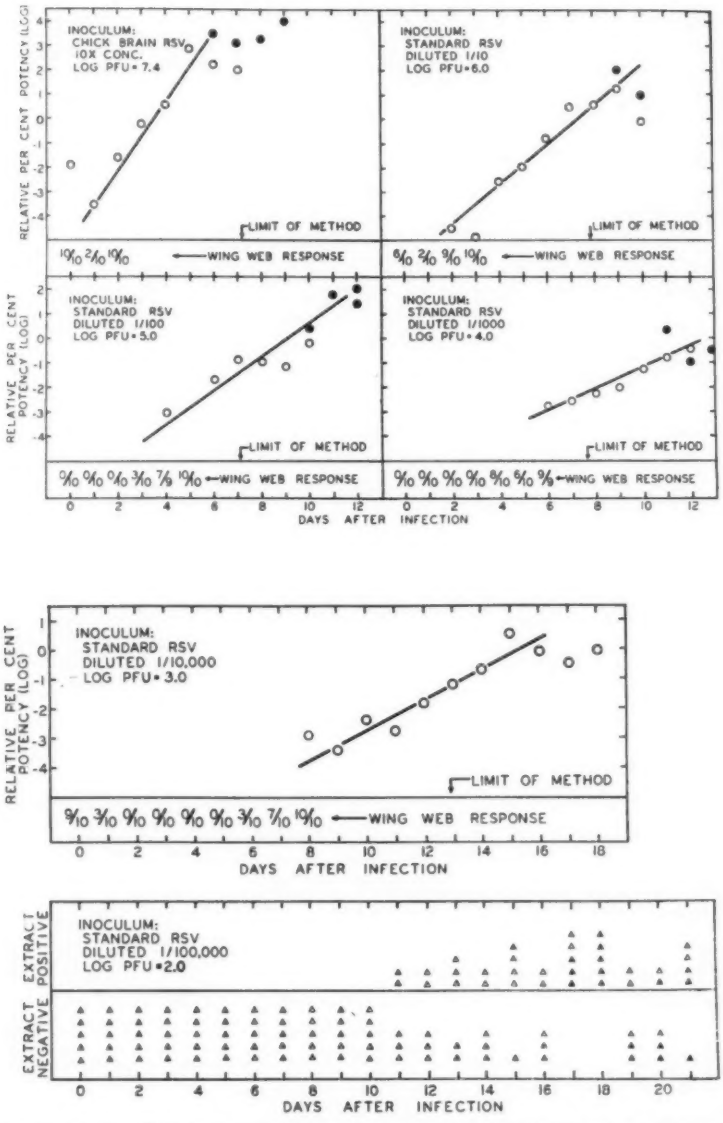
Text-figure 1 presents the cumulative mortality in percent of the 6 control groups (*top*), and in addition, the time to kill 50 percent of the chicks, expressed as 100/days, is plotted (*bottom*), showing the linear dose-response. It will be seen that, as anticipated (*1*, *2*), RSV propagated in the brain of young chicks and killed them in relation to dose, and that the time-to-death response of chicks after intracerebral inoculation of RSV was an accurate indication of viral potency.

The data presented in text-figure 2 show the viral growth curves obtained for each of the 6 different doses of RSV. The data were derived from approximately 8,000 chicks, each growth curve representing separate



TEXT-FIGURE 1.—Response of chicks (subgroup A of each group, *see* text), to intracerebral inoculation of each of 6 different doses of RSV. *Upper part* shows the cumulative mortality, in percent, in relation to infecting dose of virus. *Lower part* presents the dose-response curve expressing the time to kill 50 percent of the inoculated animals per dose of virus.

series of titrations. The chicks were inoculated with appropriate serial decimal dilutions of RSV. Beginning 5 minutes after inoculation of virus and daily thereafter, 3 to 5 chicks were killed each day and their brains removed and stored at —70° C in sealed-glass ampoules. When the birds began to die as a result of inoculation of virus, brain tissue was collected each day from both living and moribund birds. Dead birds were discarded. On the day of assay the pools of brain tissue were thawed and 10 percent homogenates of each aliquot of brain tissue were prepared as described previously (*see* "Materials and Methods"). Parallel bioassays were carried out in separate groups of chicks by two different procedures: (*a*) subcutaneous inoculation into 10 to 15 chicks per homogenate and subsequent determination of latent period to tumor production, and (*b*) intracerebral inoculation into groups of 35 to 40 chicks per homogenate and subsequent determination of time-to-death. The linear dose-response curves obtained with the standard virus provided a reference for computing relative percent potencies of the brain-tissue homogenates. All birds were examined daily for the production of tumors or for death, depending upon the route of infection. The relative potency of each brain suspension was then determined by procedures described under "Materials and Methods" [*see also* (*2*)]. The limit of sensitivity



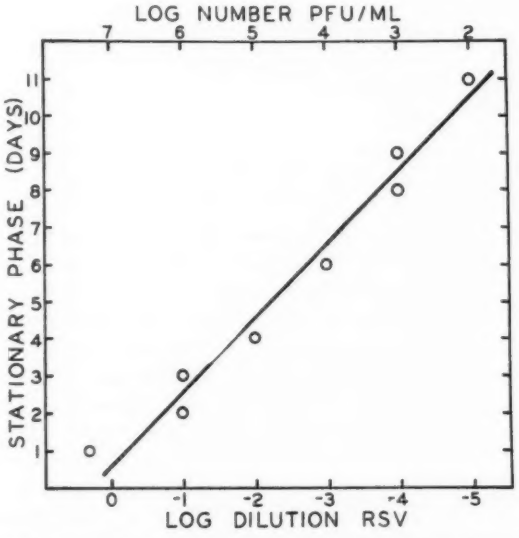
TEXT-FIGURE 2.—Growth curves of RSV after intracerebral inoculation of measured amounts of virus in young chicks. ○ = Tissue collected from apparently healthy chicks; ● = tissue collected from moribund chicks.

of the method for any given experiment was determined by the susceptibility of that particular lot of chicks to RSV.

Examination of the data summarized in text-figure 2 reveals that: (*a*) Brains removed from chicks within 6 hours after infection contained

little or no virus, which indicated that virus disappeared very rapidly from the brain. (*b*) Depending upon the original infecting dose of RSV, there occurred an "eclipse," or stationary period, during which the infective virus content of the brain remained low. (*c*) At a subsequent time, depending on the infecting dose of RSV, virus was present in relatively large amounts and continued to increase at a regular rate until it reached a peak in moribund or prostrate birds. (*d*) In chicks infected with 10,000 to 10 LD50 of virus, the peak of viral potency was comparable to that of the standard virus used for the intracerebral inoculation. However, brains removed from both apparently healthy and moribund chicks, infected with only 1 LD50 of virus, contained only moderate amounts, or amounts of virus which were too small to be measured accurately by the method of assay. Seven of 15 brains removed from individual moribund chicks on days 19 to 21, and tested separately, were noninfective.

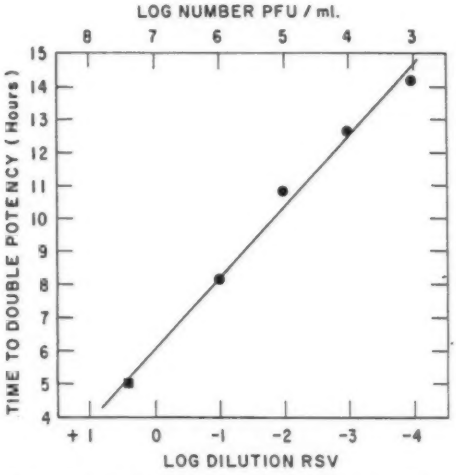
The data presented in text-figure 2 show clearly the presence of a definite eclipse period of the virus in chick brain. The length of this period was related to the infecting dose of virus. This relationship is further illustrated in text-figure 3. The points plotted as the eclipse periods of the viral growth curves represent the number of days between intracerebral inoculation of virus and the beginning of the logarithmic increase in measurable infective virus in brain homogenates. The stationary periods of the growth curve of RSV diluted 10^{-5} represent the number of days elapsing between infection and appearance of detectable infective virus only, rather than a measured quantity of virus. This qualitative criterion



TEXT-FIGURE 3.—Effect of infecting dose of RSV on the eclipse phase of viral growth curve in chick brain.

was necessary at this low dose level because, as anticipated (*2*), the variable number of infective brains per day and the low virus titers of these brains, made it impossible to obtain a quantitative estimate of relative potency.

By the use of those portions of the growth curves of RSV which showed logarithmic increases in potency, it was possible to calculate the time required for the viral potency of brain tissue to double. These data are presented in text-figure 4. The potency doubling time of the virus in chick brain was also markedly influenced by the infecting dose of RSV. Since the stability of RSV in brain tissue is not known, it has not been possible to estimate what might be comparable to generation time in bacteria.



TEXT-FIGURE 4.—Effect of infecting dose of RSV on the time to double-extractable potency in chick brain.

Distribution of Virus in the Central Nervous System After Intracerebral Inoculation of RSV

The ease with which large amounts of virus may be extracted from infected brain tissue prompted further studies for ascertaining the optimum conditions necessary for obtaining virus preparations of maximum potency and stability. It was hoped that these studies would also contribute to an understanding of the site of virus replication and of the mechanism by which virus is produced in the brain. The growth curves of RSV in chick brain show clearly that brain tissue removed from moribund birds is more potent than brain tissue removed on the same day from inoculated birds that appeared healthy at the time they were killed. Studies still in progress show that homogenates of the lung, liver, spleen, and kidney removed from moribund chicks, previously infected intracerebrally with RSV, contained amounts of virus comparable to brain tissue. It was therefore of interest to determine whether or not different

components of the central nervous system contained levels of extractable virus comparable to those found in homogenates of whole brain tissue. The results of such studies, representing a summary of data derived from several experiments, are presented in table 1. In these experiments, groups of chicks were inoculated intracerebrally into the right hemisphere with standard virus diluted 1 : 10. The indicated tissues were harvested from individual moribund birds and were assayed in embryonated eggs by the pock-counting method.

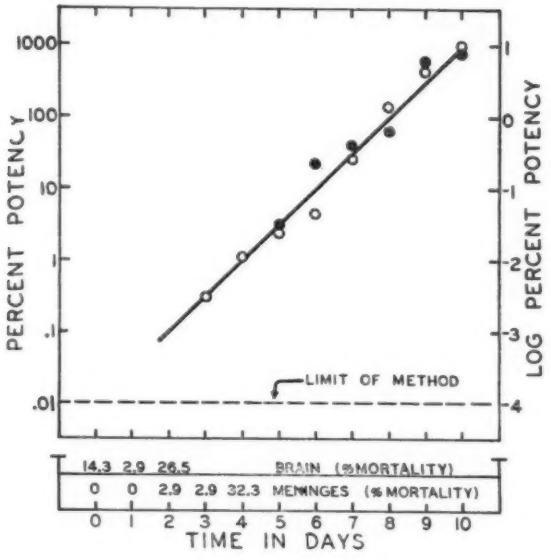
TABLE 1.—Distribution of virus in the central nervous system after intracerebral inoculation of RSV*

Chick Number	Log pock-forming unit (PFU) titer of tissue†						
	Whole brain	Whole meninges	Right cerebrum	Left cerebrum	Whole cerebellum	Spinal fluid	Spinal cord
501	6.5	—‡	6.4	6.6	6.4	—	—
428	6.5	7.3	—	—	—	4.7	6.8
429	6.8	7.2	—	—	—	3.8	6.8
430	6.1	6.1	—	—	—	3.6	—
116	6.5	8.0	—	—	—	4.5	6.7
520	6.7	7.1	6.5	6.5	6.7	5.5	7.1

*Inoculum = standard RSV diluted 1:10.
† Titers expressed as the log number of PFU per gram of tissue.
‡ Not done.

The data show that: (*a*) Homogenates of whole brains contained large and relatively uniform amounts of virus. (*b*) There was no significant difference in the level of virus from different portions of the brain itself. (*c*) Virus was present in cerebrospinal fluid and in homogenates of spinal cord in amounts smaller than or equal to those found in whole brain tissue. (*d*) Homogenates of meningeal tissue from individual birds showed levels of virus which were never less than, and usually significantly higher than, those found in whole brain tissue. It will be recalled that histological examination of several brains from moribund chicks showed no gross lesions in the brain, though an abundance of typical sarcoma cells was found beneath the meninges (*1*, *4*, *5*), and that invasion of the brain parenchyma has been recently observed (*6*). Inasmuch as the sarcoma cells in meningeal tissue could be a major site for the synthesis of RSV, the growth curve of standard virus diluted 1:10 was repeated. This experiment was performed exactly as described earlier, with the exception that both brain and meningeal tissues were harvested from 3 chicks each day and pooled, beginning 5 minutes after infection and daily thereafter. On the day of assay, 10 percent homogenates of both types of tissue were inoculated intracerebrally into groups of 35 to 40 chicks. The relative potency of each pool of tissue homogenate was determined as described previously. The results of this experiment are presented in text-figure 5. Virus disappeared rapidly from the brain and remained at low levels until the 3d day after inoculation, when the potencies of brain tissue markedly increased and continued to increase

at a regular rate. The viral content of meningeal tissue was negative or very low until the 5th day, at which time the potencies of the tissue homogenates increased at a rate indistinguishable from that of whole brain. The fact that the stationary phase of the virus for whole brain tissue was 2 days shorter than that for meningeal tissue suggests that RSV was synthesized in the brain proper. However, the exact cellular site of viral synthesis within the brain remains unknown.

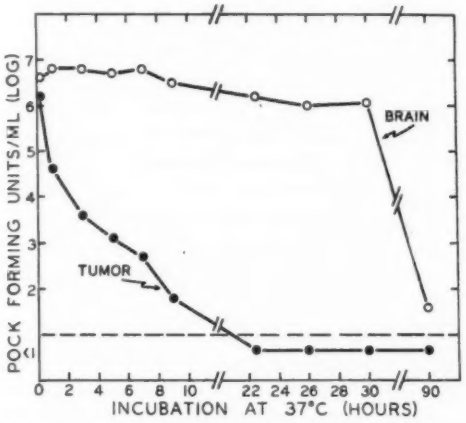


TEXT-FIGURE 5.—Comparative growth curves of RSV in brain and meningeal tissue of young chicks. ○ = Brain tissue; ● = meningeal tissue.

Thermostability of Brain-Propagated RSV

A recent report from this laboratory described the series of events that occurred during 30 serial intracerebral passages of standard RSV in newly hatched chicks (*3*). These data showed that serial passage was associated with a significant and progressive increase in the viral potencies of brain tissue. To date, a total of 64 serial intracerebral passages has been completed, and homogenates of brains from the 64th passage were approximately 30 times more potent than brain tissue from the first passage. In addition to an increase in viral potency of brain tissue, serial brain passage has resulted in an increase both in (*a*) the incidence and severity of hemorrhagic disease and (*b*) in tumor-producing potency after subcutaneous inoculation into the wing web. During these studies it was found that RSV from crude 10 percent homogenates of brain tissue was considerably more thermostable than virus contained in similar homogenates of chicken tumor tissue. When a 10 percent homogenate of a

chicken sarcoma was prepared and then assayed immediately, the log titer was 6.8. However, when the same homogenate was maintained at 4° C for more than 8 to 10 hours, the log titer dropped to <1.3. On the other hand, homogenates of brain-propagated virus maintained their original potencies for at least 2 months when held at the same temperature. The data presented in text-figure 6 show that the thermostability of brain-propagated RSV also extended to higher temperatures. In this experiment, 10 percent homogenates were prepared of a rapidly growing sarcoma and of a pool of brains harvested from chicks of the 52d serial intracerebral passage of RSV. Both types of tissue were harvested from 12-day-old chicks and both were extracted with saline containing 2 percent inactivated normal horse serum. Each homogenate was clarified by centrifuging at 2000 rpm for 20 minutes. The supernatants were then placed in a water bath at 37° C. Samples were withdrawn periodically as indicated in text-figure 6. The data show clearly that virus extracted from chick brain was considerably more stable at 37° C than virus extracted from a chicken sarcoma. After 30 hours' incubation the decrease in the potency of RSV from brain tissue was only barely demonstrable.



TEXT-FIGURE 6.—Comparative thermostability of RSV extracted from brain tissue and from tumor tissue at 37° C.

DISCUSSION

Studies on replication and growth of RSV in various experimental systems have been reported previously. In 1953 Carr (*14*) reported that, after inoculation of RSV in the leg muscle of young chicks, there occurred a 100-fold drop in infectivity 16 hours after inoculation. This was followed by a regular increase in measurable virus beginning 70 hours after inoculation. Similarly, Harris (*15*) showed that very little virus could be recovered during the 48-hour period after inoculation of RSV onto

the CAM of embryonated eggs. However, between 48 and 72 hours after inoculation, a rapid liberation of RSV occurred at a rate which paralleled the development of pocks on the CAM. Prince (*16*, *17*) extended the work of Harris during a comprehensive quantitative study of the multiplication of RSV on the CAM of chick embryos. He confirmed the existence of a stationary or "eclipse" period and, more importantly, showed that the duration of this period was directly influenced by the amount of virus inoculated. Recently, Vigier and Goldé (*18*) reported their studies on the growth curve of RSV in chick-embryo cells cultivated *in vitro*. In infected cells the virus went through an "eclipse" phase of about 2 days, followed by a growth period wherein the amount of newly formed virus increased in an apparent stepwise manner. In addition, they confirmed the observation of Rubin (*19*) that only a small fraction of infected cells became virus producers.

The studies presented in this paper show that the growth of RSV in the brains of young chicks infected with moderate to large amounts of virus is not unlike that of the non-neoplastic animal viruses. Three phases commonly observed with other viruses are also found with RSV: (*a*) A large proportion of the infective virus rapidly disappears from the tissue; (*b*) a stationary or "eclipse" phase occurs, during which the infective virus content of the tissue remains low, and (*c*) there is a progressive, regular increase in viral concentration in the tissue. However, the data reported here show a phenomenon which is at variance with common experiences with non-neoplastic viruses such as influenza virus (*20*) and pneumonia virus of mice (PVM) (*21*). This is apparent from the fact that the relative potencies attained at the peaks of the growth curves decreased with the successively smaller amounts of virus contained in the original infecting inoculums. The extent of this relationship becomes especially clear in those birds which succumbed to intracerebral infection with standard RSV diluted to a dose of approximately 1 LD50. The failure to recover any infective virus from a significant number of brains of moribund chicks inoculated with 1 LD50 or less of RSV is in sharp contrast to common experiences with many nononcogenic animal viruses. It has become common practice in most laboratories to pass stock strains of viruses at limiting dilution to insure seed pools of continued high potency and maximum homogeneity. This relationship, which apparently is unique to at least one tumor virus, confirms and extends the original observation of Bryan, Calnan, and Moloney (*8*), in a different experimental system. These investigators found that the amount of virus extractable from a given tumor was highly correlated with the amount of virus used to initiate that tumor. When sarcomas were induced with less than 1 ED50 of virus, homogenates of 24 percent of such low-dose tumors were noninfective. The data presented here show, in addition, that the stationary phase of the viral growth curve and the time to double potency after intracerebral inoculation of RSV are directly related to the infecting dose of virus.

Host responses to RSV found to be dependent on the amount of virus

contained in the infecting inoculum are listed below. It is readily apparent that all host responses to RSV which can be measured are directly related to the infecting dose of virus.

Incidence of tumors (*8, 10, 22*)
Latent period for tumor production in chicks (*8, 10, 22*) and in turkeys (*23*)
Number of pocks on CAM of chick embryos (*24, see also 13*)
Number of foci of altered cells in tissue culture (*25*)
Tumor type in chicks (*8, 26, 27*)
Rate of tumor growth in chicks (*10, 22*) and in turkeys (*23, 28*)
Virus titer of tumor (*8, 29*), and of brain tissue (*2*)
Number of virus particles per cell (*30*)
Time-to-death after inoculation of virus intracerebrally (*1*) and subcutaneously (*22*) in chicks, and intracerebrally (*28*) and subcutaneously (*28*) in turkeys
Eclipse period in chick brain (*present paper*)
Rate of viral synthesis in chick brain (*present paper*)
Incidence and severity of hemorrhagic disease and hepatic lesions in chicks (*3*) and in chick embryos (*31*)
Liver mestastases in turkeys (*28*)
Antibody response in turkeys (*28, 29*)
Incidence and rate of primary tumor regressions in turkeys (*28*)

It is of fundamental importance that an agent whose oncogenic properties have been so well documented has also been shown to resemble and to differ, depending on the dose of virus used, from the responses produced by nontumorigenic viruses. It remains equally important to determine whether or not the relationship of these various parameters of response to infecting dose is unique to RSV or whether this property is shared by other neoplastic viruses, increasing numbers of which are now being discovered.

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Prolonged Survival of Skin Homografts in Parous Female Mice [1, 2]

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SUMMARY

The survival of DBA/2 skin homografts was prolonged on postpartum BALB/c female mice parous by DBA/2 males. Survival increased with multiparity through 3 litters, but no additional increase was seen with females that had 4 or more litters. Graft survival was not prolonged on virgins or females parous by intrastrain matings. In this test system, the tolerance induced by parity appeared partial or incomplete because no permanently surviving grafts were observed among 185 test animals. After BALB/c females rejected a homograft of DBA/2 skin, their resistance to a DBA/2 tumor was tested. Normal BALB/c mice can be readily immunized against this tumor by prior inoculation of blood. As expected, the 2 control groups were immunized by the skin and no tumors grew. However, in most of those parous by DBA/2 males some degree of tolerance persisted, as evidenced by growth of the tumor. No indication of maternal immunization by the hybrid fetus was observed.—J. Nat. Cancer Inst. 25: 1405–1410, 1960.

TISSUES EXCHANGED between genetically dissimilar individuals are usually rejected as a result of an immune reaction, but the mammalian fetus may represent a naturally occurring exception to this general rule. One possible factor involved in this situation may be a suppressed ability on the part of the mother to react against the foreign antigens present in her hybrid fetus. As evidence in support of this, we have observed tolerance to a homologous tumor graft in postpartum mice parous by males of the strain in which the tumor originated. This effect increased with multiparity and was specific for the genetic constitution of the breeding male, *i.e.*, tolerance was not found in females parous from intrastrain matings or in those parous by males of a third strain unrelated to the tumor. In addition, the effect is long lasting; it is not the result of mere exposure to the male in caging, *i.e.*, separated by a wire-screen barrier, and an induced resistance can be partially abrogated by strain-specific parity (*1*).

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In the present study, evidence is presented to show that the survival of skin homografts is prolonged in specifically parous females, *i.e.*, mated to a male isogenic with the graft. These results extend our previous observations to include normal tissues and demonstrate additional quantitative aspects of the tolerance induced by parity.

MATERIALS AND METHODS

All skin-homograft recipients were strain BALB/cAnN female mice. They were divided into 3 groups as follows: One was mated to DBA/2De males to produce from 1 to 7 litters, the second was mated to BALB/c males to produce from 1 to 7 litters, and the third was not mated. Ages were matched as nearly as possible, but on an average, the females parous by intrastrain matings were oldest, and those parous by interstrain matings were older than the virgins. All litters were discarded, and on an average of 15 days after the parous females produced their last litter, females from each of the 3 groups received a single graft of DBA/2De *female* skin. In a manner previously described, full thickness grafts averaging 8 mm in diameter were transplanted into beds prepared on the right thoracic walls of the females (*2*). Bandages were removed on the 9th day, and grafts were observed daily until rejection was apparently complete.

Daily graft-survival scores were based entirely on gross appearance. Graft survival was measured in days—beginning at day "0" (the day of grafting) through the day prior to that on which eschar formation began in the last remaining area of apparently viable graft epidermis. When breakdown was considered complete, the remaining graft surface had begun to lose its normal color and texture and had become light brown or buff. In most cases, these proved to be convenient characteristic features. In a few of the grafts that survived for prolonged periods, a definite endpoint could not be determined with these criteria. These grafts underwent a prolonged period of rejection that was often accompanied by an apparent replacement with host tissue. In these, survival time was taken as either the day before that on which survival became questionable or the last day on which brown hair could be seen in graft follicles.

To test the immunity produced by skin-homograft rejection, females from each of the 3 groups that had rejected their DBA/2 skin homografts were inoculated with the genetically related Sarcoma DBA49. The interval between completion of skin-graft rejection and tumor challenge averaged 6 days. This tumor, originating in strain DBA/2, is transplantable into 70 to 90 percent of normal BALB/c mice and has been maintained in the latter strain, but such mice can be immunized against this tumor (*3*). Each female received a single inoculation of 0.025 ml of a 50 percent tumor suspension in saline subcutaneously in the right

flank, at least 1 cm posterior to the graft-bed area. The definition of tumor take was the same as that used in previous experiments (*1*).

During the experiments, a few animals developed a respiratory infection that was treated with aureomycin given orally in drinking water. The use of this antibiotic had no apparent effect on graft survival.

The data presented in the following section were analyzed by chi-square and *t* tests.

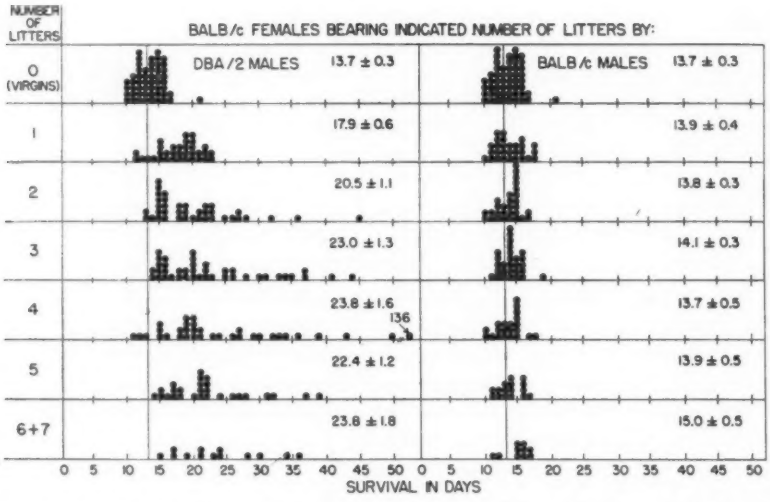
RESULTS

Before the onset of rejection, skin homografts on females parous by DBA/2 males had a better general appearance than did homografts on females of the two control groups. For example, 58 percent (107/185) of grafts on specifically parous females showed evidence of hair growth, whereas this was seen in only 4 percent of virgins (2/52) and females parous by intrastrain matings (5/132). The rate of graft rejection was generally more rapid in the two control groups, and once under way, the rejection process continued at a relatively constant rate until the entire graft was destroyed. The rejection of grafts on females parous by DBA/2 males was usually prolonged. The onset was evidenced by the beginning of eschar formation in one or more small isolated areas on the graft surface. These areas would usually slowly increase in size and coalesce until the entire graft was apparently destroyed. Sometimes, however, they would heal over so that some grafts underwent 1 or 2 cycles of scabbing and healing before their complete rejection.

The survival time of each graft is represented by a dot in text-figure 1. The survivals of skin homografts on virgin females are shown on both sides of the text-figure along with reference lines representing their mean survival (13.7 days) so that their survival can be more readily compared with that of the parous females. The survival of each graft was used in the calculations, except for one that survived for 136 days on a female with 4 litters by a DBA/2 male. This unusual survival was omitted as it might unduly bias the results.

The survival of grafts on females parous by BALB/c males was similar to that in virgins and did not increase with multiparity. A possible exception is the group that had 6 and 7 litters. Their mean survival showed a slight, but not significant ($P>0.05$ in all cases), increase over that observed in virgins and in females parous by intrastrain matings.

DBA/2 skin homografts survived longer on females parous by DBA/2 males than on virgins or those parous by BALB/c males. For example, 38 percent (71/185) of grafts on specifically parous females survived at least 22 days, while none of the grafts on virgins (0/52) or females parous by intrastrain matings (0/132) were viable at this time. Furthermore, the mean survivals of skin homografts in the groups of specifically parous females were significantly greater in comparison to those of grafts on females parous by BALB/c males for each corresponding number of litters ($P<0.01$ in all groups). In the specifically parous females, mean survival



TEXT-FIGURE 1.—Influence of interstrain and intrastrain matings on survival of DBA/2 skin homografts on BALB/c females. Each dot represents the survival time of a single graft on an individual host. Survival times, in days, are represented on the horizontal axis. The single group of virgin hosts is represented at the head of each column, and the average survival time of their grafts is shown by vertical lines, for comparison. The numbers in each rectangle refer to the average survival ± standard error.

increased with successive litters through the third litter, but no statistically significant additional increase was apparent in females with 4 or more litters. The mean survival of grafts on females with 2 litters showed an increase over primiparous females ($P = 0.04$) with a slight, but not significant ($P > 0.05$), increase in females with 3 litters as opposed to those with 2. Three or more litters produced a highly significant increase in survival over that obtained in females with 1 and 2 litters ($P < 0.001$). It was apparent that 2 or more litters were necessary to produce the maximum obtainable effect; *i.e.*, the 23-day maximum survival of grafts on primiparous females was exceeded by 50 of the grafts on multiparous females.

The results of inoculating DBA/2 tumor into females that had rejected DBA/2 skin were in conformity with previously reported observations. Upon challenge with Sarcoma DBA49 subsequent to skin-graft rejection, the tumor grew only in females parous by DBA/2 males ($89/115 = 77\%$) and not in virgins (0/18) or in females parous by BALB/c males (0/50). In specifically parous females, there was a slight indication that the percentage of animals with tumors was higher in multiparous females, but this difference was not significant. There was an apparent relationship between skin-graft survival and tumor take in that all animals, on which the skin graft persisted for 21 days or longer, grew the tumor (the one virgin female whose graft persisted for 21 days was not tested).

DISCUSSION

These results show that, within this genetic system, tolerance induced by parity is manifested with skin homografts as well as homografts of tumor, previously reported (*1*). The transplants of homologous skin yielded more quantitative information than did tumor, since grafts of normal tissues are apparently more sensitive indicators of the degree of host resistance. The mean survival of skin homografts on specifically parous females was prolonged in comparison to that of grafts on virgins and on females parous by intrastrain matings. A related observation has been made by Prehn, in that C57BL females parous by intrastrain matings exhibit tolerance to C57BL male skin grafts (*4*).

Medawar and Sparrow have reported that prolongation of skin homograft survival resulted from increased host age and not specific parity (*5*). In the present studies this was not apparent, with one possible exception. An indication of decreased immunologic response was observed in the oldest group of females parous by intrastrain matings, as manifested by a slight increase in average graft survival, but this was not significant. That the age factor played no apparent role in this test system was evidenced by the fact that, for a given number of litters, the females mated in strain were older, but graft survival was not prolonged as it was in the specifically parous females. Furthermore, there was no significant rise in mean survival time after the third litter in either group, and the average ages of the 2 control groups straddle the age of the test group.

Previous studies with a homologous tumor gave no indication of the degree of tolerance or resistance in specifically parous females. The results with skin homografts show that the tolerance is incomplete, since no permanently surviving grafts were observed. In addition, no indications of early rejection (resistance) were seen in grafts on specifically parous females, which might suggest that maternal immunization had occurred in any of these females as a consequence of having borne (BALB/c $\times$ DBA/2)F_1 hybrid young.

Parity apparently induced different degrees of tolerance in individual females. Evidence in support of this is the variation in prolonged graft survival. Furthermore, 2 or 3 previous pregnancies were necessary to attain the maximal effect. It was apparent that only a partial effect occurred in primiparous females, since the maximum survival of grafts in this group was exceeded by a significant number of grafts on multiparous females.

This phenomenon is not a special feature of the strain combination used here or of the tumor used here and previously. Experiments in progress indicate that similar observations can be made with a different strain combination, different tumor, and different relationships at the *H-2* histocompatibility locus. In preliminary experiments, the results suggest that skin grafts in a third strain combination may confirm the results reported here. The report of Prehn may also be recalled in this connection (*4*).

We have previously speculated on the role of tolerance induced by parity on tumors of chorionic origin (*6*). These tumors are unique in that they probably represent naturally occurring homografts (*7*, *8*). In a discussion of this topic, Prehn has presented evidence which suggests that the frequency of choriocarcinoma in women increases with multiparity, and, if this is the result of increased tolerance to a homologous tissue, such a relationship would be consistent with that reported in mice (*4*). Peer has found that skin homografts made from child to mother often survive for unexpectedly prolonged periods (*9*). This may indicate that tolerance induced by parity also occurs in man, but his data contain no information on the multiparity status of the women tested.

In a high percentage of specifically parous BALB/c females, a DBA/2 skin homograft did not elicit a sufficient degree of immunity to prevent the growth of a subsequent challenge with a DBA/2 tumor, and similar results were previously obtained with a prior inoculation of DBA/2 blood. These results indicate that some degree of the strain-specific tolerance persists, since these females cannot be immunized by a method that is effective in normal animals. One may speculate that this finding might, in part, account for the apparent inability of Doniach and his coworkers to immunize effectively a patient against her choriocarcinoma (*10*). However, a low degree of resistance may have been induced, since it was their impression that a beneficial effect was derived from this procedure.

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Behavior *In Vitro* of a Mouse Lymphoid-Leukemia Virus [1,2]

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SUMMARY

Maintenance of the Moloney murine leukemia virus in serial passage *in vitro* has been established. With the techniques for culture described, replication of the agent occurred in cultures of normal mouse spleen without evidence of gross cytopathic changes. The induction of leukemia in mice served to demonstrate the infectivity of tissue-culture fluids. Infected cultures continued to produce virus for extended periods without subculture. When there was an interval of 2 or more weeks between passages in normal mouse-spleen cultures, the leukemia agent could be propagated in continuous passage *in vitro*.—J. Nat. Cancer Inst. 25: 1411–1419, 1960.

DURING THE past decade, induction of leukemia in murine animals by cell-free extracts of leukemic mouse tissues has been established in several laboratories (*1–7*). The subject has been reviewed by Gross (*8*) and more recently by Moloney (*9*). Few successes have been reported concerning the maintenance of the various leukemia viruses *in vitro*.

Moore and Friend carried the Friend virus in serial passage in mouse-embryo tissue cultures (*10*). The persistence of virus was determined by animal inoculation. Irregular results were considered to be due to a low rate of virus replication *in vitro*, or to the action of an inhibitor (*11*). Attempts by Graffi and coworkers to grow their myeloid leukemia virus in Maitland and roller-tube cultures of mouse embryo, brain, bone marrow, lymph nodes, and spleen were reportedly negative. However, cell-free nutrient fluid of leukemic mouse tissue grown *in vitro* had definite leukemogenic activity (*12*). Werder and associates (*6*), working with the lymphocytic neoplasms of CFW mice, made several successful attempts to transfer serially the filterable agent(s) in a number of tissue-culture systems. Unfortunately, details are not available.

The purpose of this report is to present available information on the behavior *in vitro* of the Moloney murine leukemia virus, and to describe the techniques employed for its propagation in serial passage *in vitro*.

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[2] Presented in part at the 51st Annual Meeting, American Association for Cancer Research, April 8, 1960.
[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

MATERIALS AND METHODS

Mice.—Strain BALB/c mice from the National Institutes of Health Animal Production Section were used. They were housed in stainless-steel animal cages, 1 litter per cage, and were given Purina laboratory chow and water *ad libitum*.

Virus.—The virus used in the experiments was obtained through the courtesy of Dr. J. B. Moloney. It consisted of a cell-free extract of a pool of lymph nodes and spleens from leukemic BALB/c mice prepared by the procedure routinely used in his laboratory (*7*). One ml of extract was equivalent to 1 g of the original tissue extracted. BALB/c mice bearing the virus-induced neoplasm used for the preparation of tissue cultures were also obtained from Dr. Moloney.

Medium.—The culture medium consisted of 3 parts of human serum and 7 parts of a yeastolate basal medium. Serum was filtered with very light suction through a Selas filter candle of 015 porosity followed by sterilization by passage through a Millipore HA filter. Serums were routinely heated for 30 minutes at 56° C. The yeastolate basal medium was adapted from that described by Syverton and McLaren (*13*). It consisted of a solution of Hanks' salts, from which sodium bicarbonate and glucose were omitted. To the salt solution were added 0.1 percent Difco yeastolate, 0.25 percent glucose, and phenol red to a concentration of 0.001 percent. The solution was sterilized by the filtration steps already described for serum. Antibiotics were added in amounts of 100 units of penicillin G and 100 μg of streptomycin sulfate per ml of culture medium. Occasionally, 25 μg of aureomycin hydrochloride per ml was added to medium used for the initiation of cultures. The *p*H of the complete medium was 7.4.

Cultures.—For routine purposes, the required tissues were obtained from apparently normal mice 1 month of age. Leukemic mouse tissues were obtained from mice 3 to 4 months of age, with virus-induced leukemia. Five to 8 donor mice were killed by cervical dislocation and the desired organs—usually the spleen, thymus, or a mixture of both—were removed and placed in a petri plate in an ice bath. The tissues were minced with curved scissors and thoroughly washed at least 3 times with Hanks' salt solution. For this purpose, the tissue was suspended in the wash solution, and 10 minutes elapsed before decantation and resuspension of the sedimented tissue in the succeeding washing solution. Adequate washing facilitated sedimentation of cells after trypsinization. To disperse the cells, we suspended the washed tissue in Hanks' salt solution of *p*H 7.4, containing 0.25 percent trypsin, and agitated it mildly or pipetted it vigorously at frequent intervals during incubation for 30 minutes in a 37° C water bath. After sedimentation by centrifugation for 10 minutes at 800 rpm in an International PR–2 refrigerated centrifuge, the cells were resuspended in growth medium for a final wash. Cells were again sedimented by centrifugation in the manner described. The packed cells were suspended in 100 times their volume of growth medium when they

were derived from a leukemic animal, but in 150 times their volume when they were from a normal mouse. No bicarbonate was incorporated in the medium at this stage, nor were tubes gassed with a CO_2-air mixture. One ml of cell suspension was delivered into each of the 16 × 125 mm tubes to be used. After 3 to 4 days' incubation at 37° C, fluids were replaced with 1.5 ml of growth medium. Thereafter, fluids were replaced 3 times each week. As growth progressed, the addition of a 1.4 percent solution of sodium bicarbonate amounting to 5 to 10 percent of the volume of medium was required for adequate *p*H control.

Animal tests.—Animal tests provided the sole means for determining the presence of active virus. BALB/c mice were inoculated during the 1st day of life with 0.1 ml of culture material, usually by the intraperitoneal route. The occurrence of leukemia among inoculated mice which survived at least 8 weeks was used as the criterion for establishing the existence of virus in a given preparation. The disease syndrome was the same as that described by Moloney (7).

EXPERIMENTS AND RESULTS

Persistence of Virus in Cultured Tissues From Leukemic Mice

Monolayers of cells were prepared from pooled mixtures of thymus, spleen, and lymph nodes harvested from leukemic BALB/c mice. After several weeks' cultivation *in vitro* the cultures deteriorated. At intervals during the experimental period, samples containing suspended cell material were obtained at the time nutrient fluids were replaced. Portions of these samples were filtered through a Millipore HA filter. Both filtered and unfiltered samples were injected into BALB/c mice less than 24 hours old.

The induction of leukemia in test mice by fluids removed at successive intervals during the period that leukemic mouse tissues were maintained in culture is shown in table 1. Both the incidence of leukemia and the median time-to-death with leukemia are presented. The fact that virus was in the fluids in sufficient quantities to yield the same level of response in the test animals over the entire period of tissue cultivation suggests that active replication of virus occurred, and that a relatively high level of infectivity was maintained in the culture fluids throughout this time. Little difference in the median time-to-death with leukemia was observed between the test groups of animals given injections of unfiltered, cell-containing samples and those given filtered, cell-free materials. On two occasions in the course of similar experiments, newborn mice which had received unfiltered inoculums from 4-day-old cultures of leukemic mouse tissues died with leukemia within 3 weeks of inoculation. The leukemia in this case was undoubtedly due to the transfer of neoplastic, lymphoid cells. Such early deaths were not observed after injection of cell material from older cultures.

TABLE 1.—Leukemia in animals inoculated neonatally with fluids from cultures of tissues from leukemic mice

Period of incubation (*days*)	Fluids plus cells		Cell-free fluids	
	L/T*	Y_{50}, weeks†	L/T*	Y_{50}, weeks†
7	—	—	8/8	16.1
10	7/8	14.9	14/14	16.1
20	6/6	14.9	6/6	15.9
33	7/7	16.1	6/6	15.4

* Numerator, number of mice that died with leukemia; denominator, tota lnumber alive 8 weeks post inoculation.

† Number of weeks to 50 percent deaths with leukemia, estimated by the graphic rankit method (*14*).

Serial Passage of Leukemia Virus in Normal Mouse-Spleen Cultures

For the initial experiments to determine conditions which would permit the maintenance of the Moloney leukemia virus in serial passage *in vitro*, tube cultures were prepared from spleens of normal mice. The microscopic appearance of the cultures is illustrated in figure 1. Each of 6 tubes was infected with 0.1 ml of a 1:10 dilution of cell-free extract of leukemic mouse tissue. A similar set of tubes was inoculated with a cell-containing saline suspension of mixed, minced thymus and spleen tissue from a leukemic mouse. A third set of tubes served as uninoculated controls. Nutrient fluids were replaced three times a week. Passage was made each week, when possible, by transfer of one tenth the volume of fluids overlying the monolayer of cells to a fresh, normal mouse spleen culture. Lack of suitable fresh cultures resulted in a 5-day lag before transfer of fourth-passage fluids could be made. Each passage was checked for the presence of virus by animal inoculation when a sufficient number of newborn animals was available. Incubation of cultures was terminated after animal inoculation was completed. As indicated in table 2, the injection of animals frequently could not be made on the day passage *in vitro* of fluids was carried out. This did not interfere with the demonstration of the presence or absence of infective virus in individual passages. Since no appreciable difference between filtered and unfiltered inoculums (table 1) had been observed in the time-to-50-percent deaths with leukemia, filtration of fluids before passage was not considered necessary. Filtration was not desirable due to the possibility that virus would be lost on the filters, which would prevent the detection of low levels of infectivity in the passage fluids.

The results of 14 serial passages *in vitro* made in this fashion are presented in table 2. They indicate that the technique is inadequate for long-term maintenance of the virus. This is illustrated by the rapid decrease in infectivity during the first few passages in the series initiated with cell-free extract. The active agent was demonstrable for a longer period in the culture series initiated with leukemic tissue. However, virus disappeared in this series by the fourteenth passage.

As indicated in table 2, the cultures of the twelfth passage were retained without subculture for 90 days before animal tests were made. Although

TABLE 2.—Serial weekly passage *in vitro* of mouse leukemia virus—incidence of leukemia in test mice

Passage No.	Time to passage (*days*)	Time to animal test (*days*)	Initiated with leukemia tissue		Initiated with cell-free filtrate	
			L/T*	Y_{50}, weeks†	L/T*	Y_{50}, weeks†
		4	10/10	14. 0	12/13	18. 1
1	7	11	4/4	18. 7	9/9	19. 2
2	8	4	6/6	18. 2	7/9	23. 2
3	7	7	6/6	17. 2	0/3	—
4	12	15	6/6	19. 2	1/7	—
		3	7/7	20. 2	0/9	—
5	8	10	4/5	17. 4	0/7	—
6	7	—	—	—	—	—
7	9	9	7/7	17. 4	0/9	—
8	7	7	8/8	15. 2	0/7	—
9	7	—	—	—	—	—
10‡	7	7	8/8	16. 7	0/6	—
11	7	7	7/7	16. 1	0/7	—
12	7	90	11/11	16. 9	0/9	—
13	7	—	—	—	—	—
14	7	7	0/6	—	0/7	—

*Numerator, number of mice that died with leukemia; denominator, total number alive 8 weeks post inoculation.

†Number of weeks to 50 percent deaths with leukemia, estimated by the graphic rankit method (*14*).

‡Animals received filtered tenth-passage culture fluids.

the cultures appeared static during this time, this was not due to a lack of growth of the cells. Many cells became detached from the glass surfaces of the culture tubes and were lost with the fluid changes which were made 3 times each week. The growth of new cells compensated for this loss. At the end of the 3-month period, newborn animals were inoculated with unfiltered culture material. Leukemia was induced in animals only by those cultures known to have received an active inoculum (table 2). It is evident that such apparently static cultures can serve as a continuing source of virus, available for laboratory use at each change of culture fluid.

The degree of partial success achieved in the foregoing experiment suggested that a longer incubation period might be required between passages for maintenance of the virus *in vitro*. Accordingly, 2 additional cell-free passage series were initiated in which the intervals between passages were 1 week and 2 weeks, respectively. The same pool of cell-free extract of leukemic mouse tissue was employed that had been used in the previous passage experiment. Each of a set of tubes was inoculated with 0.1 ml of undiluted extract. After the 7th day of incubation, one tenth the volume of fluids overlying the cultures was passaged to fresh, normal mouse-spleen cultures, which thereby initiated a weekly passage series. The original tubes were incubated for another 7 days, and passage of fluids was made similarly to fresh, normal mouse-spleen cultures, which thus initiated the 14-day passage series. Thereafter, fluids from the latter cultures were transferred to fresh spleen tubes every 2d week. Control cultures were passaged at the same time as infected cultures.

The results in table 3 show successful maintenance of virus with a 14-day interval between passages, but failure with the 7-day interval, as in the preceding experiment (table 2). No leukemia has thus far been observed in animals inoculated with the control materials.

TABLE 3.—Effect of interval between subcultures on maintenance *in vitro* of mouse leukemia virus—incidence of leukemia in test mice

Initiated with cell-free filtrate (passage and test every 7 days)			Initiated with cell-free filtrate (passage and test every 14 days)		
Passage No.	L/T*	Y_{50}, weeks†	Passage No.	L/T*	Y_{50}, weeks†
3	0/6	—	1	7/8	19.0
5	0/5	—	2	8/8	15.4
6	0/8	—	3	6/6	15.2
7	0/7	—	4	—	—
10	0/7	—	5	7/8	19.2

* Numerator, number of mice that died with leukemia; denominator, total number alive 8 weeks post inoculation.
† Number of weeks to 50 percent deaths with leukemia estimated by the graphic rankit method (*14*).

Storage of Infected Tissue Cultures

Frozen cultures of the tenth weekly passage (table 2) and of the fourth biweekly passage (table 3) were thawed and tested after 5 months' storage in a CO_2 ice box. Tube cultures prepared from pooled thymuses and spleens of normal mice were inoculated with 0.15 ml aliquots of undiluted, thawed culture material. Thereafter, passage to fresh cultures was made every 3d week. At the same time, newborn mice received injections of both filtered and unfiltered culture fluids to test for the infectivity of the passage inoculum. The animal responses are available at the time of this writing for the first 6 passages. They show the same high levels of infectivity observed with the unfrozen cultures of the same series. The filtered fluids from each passage appeared to be as potent as the comparable unfiltered fluids for the induction of leukemia in test animals.

DISCUSSION

Tissue cultures prepared as described produced monolayers of a mixed-cell population with a predominance of cells of the ameboid type. The appearance of the established cultures was the same whether prepared from spleen or thymus, or from mixture of spleen, thymus, and lymph nodes. A few attempts were made to maintain cultures prepared from leukemic mice for long periods. In each instance, gradual deterioration led to their loss within 5 or 6 weeks. A greater degree of success has been experienced with normal mouse spleen *in vitro*. One such uninfected culture has been maintained and passaged as a mixed-cell population for about 1 year. Normal mouse-spleen cultures inoculated with infective tissue-culture fluids were retained without subculture for 3 months. They were terminated at this time due to a progressive overgrowth of

ameboid cells by other cells. By animal inoculation, the leukemia agent was shown to have persisted in these cultures throughout 3 months of cultivation.

No difference in appearance was observed between infected and noninfected cultures. The presence of virus was detected by animal response. This involved an average induction period of about 3 months before the earliest deaths with leukemia began to appear. Evidence of virus-induced leukemia was never observed in animals within 8 weeks of injection of an active inoculum. For this reason, deaths which occurred within this interval were considered nonspecific. The median time-to-death with leukemia for the various test groups of animals was estimated by the graphic rankit method (*14*). The median values for animals inoculated with fluids from cultures of leukemic animal tissues ranged from 15 to 16 weeks, as shown in table 1. Comparatively higher median values of 15 to 23 weeks were obtained with animals inoculated with material from infected cultures of normal mouse spleen (tables 2 and 3). Moloney (*7*) presented data obtained in mice indicating an inverse relationship between the dose of virus inoculated and the median time-to-death with leukemia. Comparison of the values obtained for animal response to tissue-culture inoculums with Moloney's data suggests that more potent virus preparations are obtained by the extraction of tissues from leukemic animals. However, the tissue-culture system provides a continuous supply of infective material readily available in quantity each time nutrient fluids are replaced.

Neoplasms other than leukemia were not observed in mice given injections of tissue-culture material infected with the Moloney agent. The absence of cytopathic changes in infected cultures gives strength to the conclusion that polyoma virus (*15*) is absent and in no way involved in the leukemic response of the test animals.

The data in table 2 show that passages originated with leukemic tissue harbored the virus through a greater number of passages than did those begun with a cell-free extract. The relationships of dose, virus reproductive cycle, and number of susceptible cells responsible for these results would be difficult to determine. The observations presented show that the Moloney leukemia virus, as a cell-free preparation, will infect and reproduce in cultures of mouse spleen. If a sufficient interval is maintained between passages, successful transfer to fresh mouse-spleen cultures may be made by simple inoculation of infected nutrient fluid. Once infected, cultures continue to produce virus as long as they are maintained. Infected fluids may be stored in a dry-ice chest and used to infect mouse-spleen cultures at a later date.

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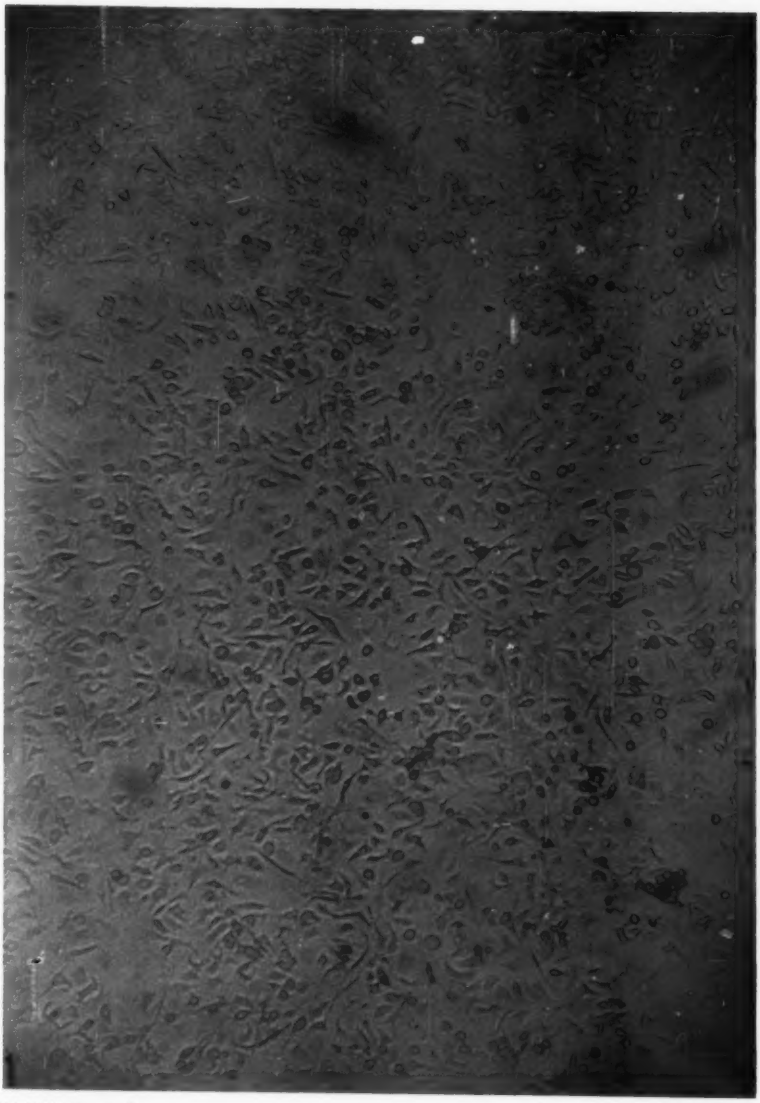
FIGURE 1.—Uninfected tube culture of mouse spleen. Unstained living culture. Infected cultures have same appearance. × 75



Virus of Avian Myeloblastosis. XVIII. Fixation of Myeloblasts and Phosphatase Activity of Loci of Virus Synthesis (Viroplasts) [1, 2]

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SUMMARY

Further work is reported on the phosphatase activity of the intracellular bodies (viroplasts) of myeloblasts from tissue cultures established with the primitive cells from the circulating blood of chickens diseased with avian myeloblastosis virus. Evidence obtained earlier indicated that the viroplasts are the specific sites of the synthesis of myeloblastosis virus, and that the viroplasts may be derived by virus infection of the precursors of granules developing in maturation of the myeloblast normally through the granulocytic series of white blood cells. Previous studies revealed that viroplasts and granules of normal myelocytes exhibited strong activity to dephosphorylate adenosine triphosphate. The present study shows that a parallel reaction occurred with both normal granules and viroplasts when inosine triphosphate was used as substrate in the Wachstein-Meisel procedure. Analogous tests with adenosine and inosine diphosphates gave slightly less pronounced reactions with granules of myelocytes, but those seen with the viroplasts were indistinguishable from results obtained with the triphosphates. Studies with adenylic acid, glucose-6-phosphate, and glycerophosphate, and tests for acid and alkaline phosphatase were entirely negative with both normal granules and viroplasts. Myeloblasts grown in culture medium containing 5-methyl tryptophan developed many large viroplasts concurrent with inhibition of cell growth, and did not lessen the rate of virus liberation by the cells. These viroplasts exhibited typical adenosinetriphosphatase activity. Methods for fixation of myeloblasts were improved by development of a practical sequence of quenching in liquid nitrogen, dehydration *in vacuo* at low temperatures, and fixation with formaldehyde vapor by which structural artifacts related to subsequent treatment can be controlled.—J. Nat. Cancer Inst. 25: 1421–1449, 1960.

EARLIER REPORTS described the results of studies (*1–4*) of the ultrastructure of the myeloblasts which are the cells of neoplastic traits characteristic of avian myeloblastic leukemia (*5*). Electron micrographic

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examination of thin sections of myeloblasts immediately after removal from the circulating blood of the diseased chicken revealed remarkably little evidence of the etiologic agent in association with the myeloblast. Within a few hours after such cells were established in tissue culture, however, there was observed the beginning development of a series of cytoplasmic bodies which have appeared to be (*4*) specific loci of virus synthesis and which participate in the elimination of the agent from the diseased cell.

Because of their size and optical characteristics, the specific structures designated as viroplasts were easily resolved and identified in the phase-contrast microscope (*4, 6*). By virtue of these properties, it has been possible to undertake a systematic study by cytochemical techniques of the constitution and reactions of the viroplasts observed by phase-contrast microscopy. It has been noted that these structures exhibit strongly the activity to dephosphorylate adenosine triphosphate. This conformed with previous evidence (*7*) of an unusually high content of this enzyme in the myeloblasts of myeloblastosis. Of further interest, the virus of myeloblastosis in purified preparations carries with it the same enzyme activity (*8–10*). The most significant outcome of the experiments was the evidence (*4, 6*) that possibly the viroplasts originated from precursors of the organelles from which are developed the granules of the myelocytic series of cells in the normal bird.

Within the limits of available techniques, the question of the constitution of the viroplasts was studied to obtain further information on the synthesis of the myeloblastosis virus. A major problem has been the development of techniques for fixation of the myeloblasts. For application of the cytochemical methods, this problem is important not only in relation to preserving the reactions of the presumably normal organelles of the cells but also for identifyng those of the virus-associated structures under test. Rapid freezing of the cells has accounted for much progress in this area (*4*), and studies of the process are continuing. This paper describes the status of the fixation procedures used in the demonstration of the reactions of the cytoplasmic structures of normal myeloblasts and myeloblastosis virus-diseased cells to a variety of phosphate substrates other than adenosine triphosphate.

MATERIALS AND METHODS

Parallel studies were made on: 1) cells from the bone marrow of normal chickens, 2) myeloblasts immediately after harvest from the circulating blood of chickens with myeloblastosis, and 3) myeloblasts from tissue cultures of the cells after various intervals *in vitro*.

The cells were photographed by phase-contrast microscopy, at which time an index was obtained of their position on the slide. After fixation and the application of the various staining procedures, the same cells were relocated and rephotographed. The cells were obtained from the

inbred White Leghorn chickens of line 15 developed (*11*) at the Regional Poultry Research Laboratory, East Lansing, Michigan.

Myeloblasts for direct examination and for preparation of tissue cultures were obtained, as previously described (*7*, *12*), from the circulating blood of chickens diseased with the BAI strain A virus (*13*). Tissue cultures were prepared in the usual way (*7*, *12*) in a medium of equal parts chicken serum and medium 199 (*14*), supplemented with glucose in 0.5 percent concentration, B vitamins in the same concentrations as those of Eagle's medium (*15*), and folic acid in the concentration of 0.8 mg per liter. Fifty units of penicillin and 50 μg of streptomycin per ml of medium were used. In one series of experiments, 5-methyl tryptophan was added to the medium in a concentration of 10 mmoles per liter.

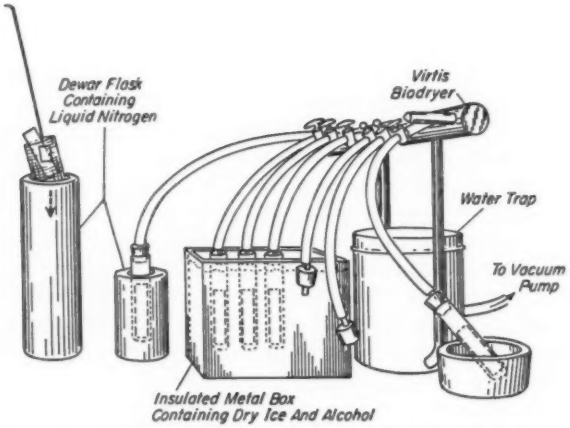
Bone marrow was taken from the femur and tibia of normal chickens, 2 to 3 weeks of age, after exsanguination. The material was suspended in a mixture of equal parts of medium 199 and normal chicken serum (*3*). Fat and clumps of tissue were removed with a curet, and mounts were made of the suspended cells.

To prepare cell mounts we deposited a drop of cell sample on a *new* coverslip which had been cleaned by wiping with lint-free paper. An acid-cleaned glass slide was carefully lowered onto the drop, so that one corner of the coverslip projected over the edge of the glass slide. With the proper volume of sample, the spreading fluid did not cover the entire area between slide and coverslip.

The specimen was examined first by low-power *dark-field* microscopy to locate areas where the cells were slightly flattened but not disrupted. Selected fields were photographed by phase contrast and indexed with a slide locator (The England Finder, Eric Sobodka Co., New York, N.Y.). After the photographs were made, usually of several fields, the slide was then placed in a suitable small wire-gauze carrier (*see* text-fig. 1), and quickly dipped into liquid nitrogen. The least possible time between photography of the living cells and quenching in liquid nitrogen was allowed in order to minimize the displacement of intracellular structures by plasma streaming. When the slide had been in liquid nitrogen for at least 30 seconds, the coverglass was rapidly pried off by means of the projecting corner of the coverglass.

From this point, one of two procedures was employed, depending on the object of the experiment.

For *dehydration and formaldehyde fixation*, the slide, after removal of the coverslip, was transferred as quickly as possible to a test tube (32 mm outside diameter × 145 mm) immersed in liquid nitrogen (*see* diagram of text-fig. 1), in which the air temperature was about −165° C. This tube was then connected to a Virtis Biodryer with a pump which had been evacuating for at least 15 minutes. After approximately 3 minutes, the test tube was transferred from the liquid nitrogen in the Dewar flask to an insulated metal box containing a mixture of dry-ice and alcohol. Evacuation was continued at this temperature (−80 to −70° C) for 1 hour, and at room temperature for 1 hour after the tubes were removed from the



TEXT-FIGURE 1.—Arrangement for freeze-drying myeloblasts on glass slides. Initial freezing is effected in the first Dewar flask, and the vacuum is first applied with the slide in the tube immersed in nitrogen in the second flask. *At right*, the tube containing the slide rests in an empty vessel to complete drying at room temperature.

dry-ice-alcohol mixture. In this period the temperature increased from −70 to 16° C. The vacuum was then broken, and the slides were stained with or without fixation. For fixation, the slides were placed in a Coplin jar, which contained just enough formalin to cover the bottom, and left for 2 minutes with the cover closed. The slides were then removed from the jars and washed in tap water.

Freeze-substitution was accomplished, after removal of the coverslip, by transfer of the slides directly from liquid nitrogen to acetone at −75° C for 5 minutes, then to a change of acetone for 5 minutes at the same temperature, and finally to acetone at room temperature for 5 minutes. The slides were air-dried.

Cytochemical analyses were made with the following substrates: 1) disodium adenosine triphosphate · $3H_2O$, 2) disodium adenosine diphosphate · $3H_2O$, 3) adenylic acid (adenosine-5′-phosphate), 4) disodium inosine triphosphate · $7H_2O$, 5) disodium inosine diphosphate · $3H_2O$, 6) barium glucose-6-phosphate · $7H_2O$, and (7) sodium glycerophosphate. The substrates were in the same molar concentration used for adenosine triphosphate in the Wachstein-Meisel procedure.

The determinations for the presence of enzymes active in the dephosphorylation of these substrates were made with the procedures of Wachstein and Meisel (*16*); half the concentration of lead nitrate suggested by these authors was used. This change did not lessen the intensity of the reaction but eliminated much of the nonspecific lead deposit which otherwise sometimes obscured the cells.

Acid and alkaline phosphatase studies were made by the metal-precipitate method (*17, 18*) and the azo-dye procedure (*19, 20*).

Electron micrographs of thin sections of various materials under study were made as already described (*4*).

RESULTS

A large part of the present work has been devoted to the development of a sequence of procedures suitable for the identification of cytochemical reactions specific to the bodies concerned with the response of the myeloblast to the myeloblastosis virus and to structures involved in the response. Well-recognized principles of quick freezing were used as they offered the best opportunities for comprehensive studies. The problem was to find the proper methods of treatment of the material after it was quenched in liquid nitrogen. Exploratory observations revealed not only the influence of the various procedures but also certain properties of the cells which were useful in the interpretation of final results.

Freeze-Substitution vs. Freeze-Drying Followed by Fixation With Formaldehyde

It has been observed (*4*, *6*) that the granules of the myelocytes and granulocytes of normal chicken bone marrow exhibited adenosinetriphosphatase activity, as demonstrated by use of the Wachstein-Meisel procedure. This reaction occurred (*4*) when the cells were fixed with formaldehyde vapor after freezing and drying as demonstrated in figure 1. The deposits of lead in figure 1B are clearly associated with certain of the granules of the myelocytes identified in the phase-contrast photograph of the living cells of figure 1A. As noted before (*4*), not all the granules seen by phase contrast exhibit enzyme activity. This does not necessarily signify a difference in principle between the various granules, but more likely is a variation dependent on the concentration of enzyme related to the stage of maturation of the granules. This is supported to some extent by the closer correlation of granules visible in phase contrast with those staining in the granulocytes. In control studies similar cells were treated by the same procedures, except that adenosine triphosphate was omitted from the Wachstein-Meisel reagent. Under these conditions, no trace of lead deposit was seen.

A result of much interest was encountered when cells of the normal bone marrow were stained for adenosinetriphosphatase activity after treatment by the *freeze-substitution* technique with acetone. The results showed, as exemplified in figure 2, that the granules seen by phase contrast, figure 2A, took practically no stain, figure 2B. Instead, the sites of the granules were represented, for the most part, by clear areas in which there was no evidence of lead deposit. This was not a completely uniform appearance, however, since some bodies visible in phase contrast, particularly in the cells containing a large number of small granules, did stain as seen, also, in figure 2B. It was not entirely clear, though, that the stained structures corresponded wholly to granules. In most of the cells treated by the freeze-substitution procedure, there was seen a dark background reaction in the Golgi field and around the vacuoles. The studies yielded no evidence for judgment of the basis for the failure of the granules to stain after treat-

ment with acetone. Obviously, enzyme may have been extracted or inactivated. Nevertheless, it is important to note at this point that the *adenosinetriphosphatase activity of the viroplasts, the structures of virus synthesis in the myeloblast, was not altered by freeze-substitution.* This marks a significant difference between the enzyme activity of the normal structures and that of the bodies concerned with the virus disease.

Influence of damage.—Much care was required in the preparation of the myeloblasts in a physical state suitable for examination on the slide. The cells in suspension are spherical or spheroidal and remain so without flattening on the glass surface. Under these conditions, little detail of their internal structure can be seen by the methods used thus far. Therefore, it was necessary to flatten the cells by pressure of the coverslip. Unfortunately, the internal structure of the cells is easily damaged by too much pressure, so that the reaction of the organelles is substantially altered, particularly that of the mitochondria. In undamaged cells, these structures exhibit no adenosinetriphosphatase activity. With damage, however, most of the mitochondria identifiable by phase contrast do stain with the Wachstein-Meisel reagent as illustrated in figure 3. This phenomenon may greatly complicate the interpretation of the results of the studies on the enzyme activity of the viroplasts.

Somewhat similar effects occurred when the cells were *frozen and dried but not fixed* before staining, as shown in the pair of myeloblasts from tissue culture in figure 4. The viroplasts seen in figure 4A produced a very strongly positive adenosinetriphosphatase reaction, but so, too, did the mitochondria. There was also a conspicuous positive reaction adjacent to and frequently extending into the central portions of the Golgi area. The reaction appeared to differ morphologically from that seen in the mitochondria, and no mitochondria are seen in the Golgi field in the phase-contrast picture of the living cell. This positive reaction in the general region of the Golgi structures sometimes formed an indistinct, completely irregular network, and sometimes it was even more diffusely spread. The reaction was frequently associated with the border of vacuoles and often with the cytoplasmic and nuclear membranes. In the cells of the normal bone marrow not fixed with formaldehyde after freeze-drying, the granules of the more mature cells of the granulocytic series gave no adenosinetriphosphatase reaction. A strong reaction was seen in certain cells which contained many small granules of uniform size. These are the same cells that retain enzyme activity after freeze-substitution (fig. 2B). Under these conditions, the myeloblasts in normal bone marrow were similar in behavior to myeloblasts from tissue culture, except for the absence of viroplasts.

Substitution of Other Phosphate Substrates for Adenosine Triphosphate in the Wachstein-Meisel Reaction

Inosine triphosphate.—The earlier demonstration of a positive adenosinetriphosphatase reaction in association with the granules of cells of the

normal bone marrow and with the viroplasts of myeloblasts from tissue culture suggested the probability that analogous results would be obtained by use of inosine triphosphate as substrate. This was the more likely since the myeloblastosis virus exhibits enzymatic activity with inosine triphosphate (*21*) in parallel with that exerted on adenosine triphosphate. That such was the case is demonstrated in figures 5 and 6. Figure 5A shows the phase-contrast picture of cells from normal bone marrow. The same cells are seen in figure 5B after freezing, dehydration, formaldehyde fixation, and treatment with the Wachstein-Meisel reagent when inosine triphosphate was the substrate. The granules of the myelocytes or granulocytes seen in figure 5A are heavily marked by the deposits of lead revealed in figure 5B. This result was similar to that observed with adenosine triphosphate as illustrated in figures 8 and 9 of (*4*) and figures 1A and B.

The results obtained in an analogous experiment with *myeloblasts* from tissue culture, figure 6, showed that the viroplasts evident by phase contrast, figure 6A, were heavily marked by deposits of lead as illustrated in figure 6B. With these cells, the results obtained with inosine triphosphate have been identical with those previously observed with adenosine triphosphate.

Adenosine diphosphate.—Figures 7 and 8 show the results obtained by use of adenosine diphosphate as substrate. By application of the same procedures used with inosine triphosphate, a definite though moderate staining was observed, figure 7B, in many of the granules of the myelocytes of normal bone marrow seen by phase contrast in figure 7A. The reaction was notably less than that occurring with either of the triphosphates. The stained bodies were apparently much smaller than the granules, many of which exhibited no deposit of lead. Such was not the case with the viroplasts of tissue-culture myeloblasts, figure 8, which were well stained (fig. 8B).

Inosine diphosphate.—When inosine diphosphate was used as substrate with normal bone marrow and with myeloblasts from tissue culture, the results were identical with those seen with adenosine diphosphate. The granules of the normal myelocytes showed a moderate reaction to the stain similar to that indicated in figure 7 with the use of adenosine diphosphate. The reaction of the viroplasts of the tissue-culture myeloblasts with inosine diphosphate was strong and comparable to that seen in figure 8 with adenosine diphosphate.

Adenylic acid, glucose-6-phosphate, and glycerophosphate.—Investigations with these substrates yielded entirely negative results, as documented by the photographs of myelocytes of normal bone marrow, figure 9, and of myeloblasts from tissue culture, figure 10, in a study with adenylic acid. Figures 9B and 10B show that neither the granules, figure 9A, nor the viroplasts, figure 10A, exhibited any reaction. The results with all these substrates were identical.

Tests for Acid and Alkaline Phosphatase

No evidence was seen of positive reaction by either the granules of the normal cells or the viroplasts. This is exemplified by the results obtained in the test by the Gomori metal-precipitate method for alkaline phosphatase in tissue-culture myeloblasts which are shown in figure 11.

Viroplasts in Myeloblasts Grown in Presence of 5-Methyl Tryptophan

A rather remarkable phenomenon has been encountered in a study of myeloblasts grown in tissue culture to which the amino acid analogue 5-methyl tryptophan had been added.

The 5-methyl tryptophan exerted a strong inhibiting effect on cell growth. At the beginning of the experiment, the concentration of myeloblasts in one series of cultures was 3.96×10^7 cells per ml, and at the end of the 8-day period of study, the cell count was 2.88×10^7 per ml. In contrast, the cells of the control cultures grew well, as indicated by a generation time (*12*) of 3.1 days during the same period.

In this interval, however, no influence was exerted on the rate of virus release by the cells. The mean rate of virus liberation in the control cultures was 31.1 particles per cell per hour in comparison with the rate of 39.2 particles per cell per hour in the cultures containing 5-methyl tryptophan.

With these results, it was surprising to observe the great increase in the number and size of viroplasts in the myeloblasts of the cultures containing the inhibitor. The appearance of a typical myeloblast from a culture exposed for 6 days to 5-methyl tryptophan is illustrated by phase contrast in figure 12. In this cell the viroplasts, many of relatively large size, are scattered throughout the cytoplasm.

Tests of these myeloblasts for adenosinetriphosphatase activity showed that the bodies with the appearance of viroplasts in figure 12A gave strongly positive reactions on treatment with the Wachstein-Meisel procedure, figure 12B.

Electron micrographs of thin sections of cells from the same cultures revealed, figure 13, the profusion of large viroplasts of typical appearance. The number of unequivocally identifiable virus particles visible in the viroplasts was very small, as seen in the picture. Frequently, many circular images suggestive, possibly, of virus in the early process of formation, were evident, but their significance could not be assessed. In general structure, the myeloblasts did not differ from those of the cells of the control cultures.

DISCUSSION

The sequence of procedures for preparation of myeloblasts for cytochemical studies represents an adaptation of well-recognized (*22*) principles specially applicable to the material used. Several aspects of the tech-

niques were of critical importance in the portrayal of the cell structures and their reactions and the reduction of artifacts.

An outstanding difficulty encountered in the study of the myeloblast is the inability of the cell to flatten on a glass surface. This must be accomplished, nevertheless, if the cell structures are to be revealed in the living state. Although several methods, including centrifugation, have been applied, a desirable degree of success has been attained only by the pressure exerted by a coverslip. Difficulties also arise relative to the following: the control of pressure by use of a suitable volume of sample; the choice for study of cells undamaged by excessive flattening; the removal of the coverslip after freezing without warming of the preparation; and the loss of indexed cells which adhere to the coverslip. As evident from the results, however, the exercise of care at each step overcomes the impediments satisfactorily.

While liquid nitrogen is not regarded as the ideal quenching material, its advantages with respect to availability, cheapness, and nonexplosive properties have recommended its continued use. It has been our objective to maintain the structural integrity of the cells until complete dehydration was effected, and this was easily accomplished with the simple equipment shown in the diagram of text-figure 1. A critical manipulation was the removal of the coverslip, which we effected simply by raising the slide out of the nitrogen and quickly prying the coverslip away with the finger.

The results obtained by application of the sequence of quenching, dehydration at low temperature, and formaldehyde fixation mark a considerable advance over those obtained with previous methods. In the study of the phosphatase systems, it has become possible to distinguish with much assurance between artifacts based on altered reactions of the organelles normal to the myeloblast and the bodies specific to the virus-diseased cell. By this means it has been possible to exclude phosphatase activity by the mitochondria in the diseased cell which was frequently observed in the initial studies. This is important because in the myeloblast the mitochondrion is the only organelle comparable in size to the viroplast. Use of the technique likewise provided a basis for the disclosure of adenosinetriphosphatase activity in the mitochondria of cells damaged by too much pressure, or by treatment of the cells, in the staining procedures without fixation with formaldehyde after dehydration *in vacuo* at low temperature. This constitutes a cytochemical corroboration of the observations by others (*23*, *24*) that aged mitochondria or mitochondria damaged by a variety of methods exhibit adenosinetriphosphatase activity, while intact mitochondria do not. The complete absence of adenosinetriphosphatase reaction in the normal myelocyte, except in bodies identified as granules, greatly strengthens the interpretation of the relationship of the enzymatic activity to the virus-specific bodies or viroplasts in the diseased cell.

Another criterion for a specific relationship was founded on the results obtained by freeze-substitution, and after freeze-drying without fixation

with formaldehyde. When these techniques were used, the granules of the myelocytes exhibited no enzymatic reaction, whereas the viroplasts were as active as after freeze-drying *in vacuo* and fixation with formaldehyde. Such behavior might be due to inactivation of the enzyme by acetone, which would be indicative of a considerable difference between the enzymes, *per se*, of the normal granules and those of the viroplasts. Another possibility is concerned with differential solubility or extractibility of the enzymes of the normal and disease-associated structures related either to the properties of the enzymes themselves, or to those of the enzymes in association with other materials, possibly of lipide or lipoprotein constitution. While the biochemical basis for the difference is subject only to speculation, it seems quite evident that the adenosinetriphosphatase of the granules differs greatly either in constitution or in state from that present in the viroplast.

The evidence thus far accumulated indicates strongly that the granules of normal myelocytes and the viroplasts of the myeloblast contain an adenosinetriphosphatase in a free and active state. That the behavior is not due to nonspecific phosphatases has been shown by means of thorough tests with the other phosphate substrates described in this report. The reaction of the viroplasts with inosine triphosphate was not surprising, since the myeloblastosis virus exhibits inosinetriphosphatase activity (*21*). The dephosphorylation of adenosine and inosine diphosphates, however, could not be correlated with the properties of the virus itself, which exhibits no activity to react with adenosine diphosphate (*10*). Moreover, it was of interest to learn that the granules of the normal myelocytes likewise showed inosinediphosphatase activity similar to that observed with adenosine diphosphate.

These properties raise the question of the reality of the presence of the two diphosphatases in the granules and the viroplasts. Several explanations may be offered. There might be assumed the presence in or near the granules or viroplasts of a kinase, such as myokinase, capable of converting adenosine and inosine diphosphates (added in the Wachstein-Meisel procedure) to the respective triphosphates. Thus the reaction observed in the granules and viroplasts with the diphosphate substrate could be due to the exertion of activity of the triphosphatases on the triphosphates formed in this way from the diphosphates. This possibility seems remote, however, since it has been observed (*25*) that, although myokinase activity may convert adenosine diphosphate to the triphosphate, the analogous reaction does not occur with inosine diphosphate. For this reason, it seems safe to assume that such a mechanism does not explain the evidence of inosinediphosphatase activity in the normal granules or viroplasts and suggests, by analogy, that it provides no explanation of the reaction with adenosine diphosphate as substrate.

Another explanation of the apparent diphosphatase reactions might be based on the possible presence in the structures of a polynucleotide phosphorylase capable of polymerizing the diphosphates to polynucleotides with the liberation of phosphate as a product of the reaction. There

is no specific evidence, at the moment, either for or against this possibility.

The existence of adenosine- and inosinediphosphatases in the viroplasts may bear on the mechanism of incorporation of the triphosphatases in the myeloblastosis virus particles. If there were only triphosphatases in the viroplasts, it might be assumed that the enzymes were adsorbed on the virus particles during their stay in these bodies. The presence of the diphosphatases in concentrations high enough to give reactions with the Wachstein-Meisel procedure provides some evidence against this possibility. Under these conditions, it would have to be assumed that adsorption of adenosinetriphosphatase would be specific to the exclusion of adsorption of adenosinediphosphatase which is not present in the virus in purified preparations.

The greatly increased number of large viroplasts in myeloblasts in cultures containing 5-methyl tryptophan is a phenomenon of much practical and, probably also, fundamental interest. It has been recognized that the factors governing cell growth and virus synthesis do not act entirely in concert, which was emphasized in this experiment by the reduced rate of cell proliferation without change in the rate of virus output per cell per hour. The basis for the increase in the number of viroplasts and their large size is not known. It is evident that they are closely similar to the viroplasts of myeloblasts in ordinary tissue culture. For this reason the phenomenon provides increased opportunities and material for study of the biochemical characteristics of the viroplasts. Moreover, the action of this amino acid analogue may furnish one more means to approach the problem of the origin of the viroplast and of the processes within it concerned with virus synthesis.

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PLATES

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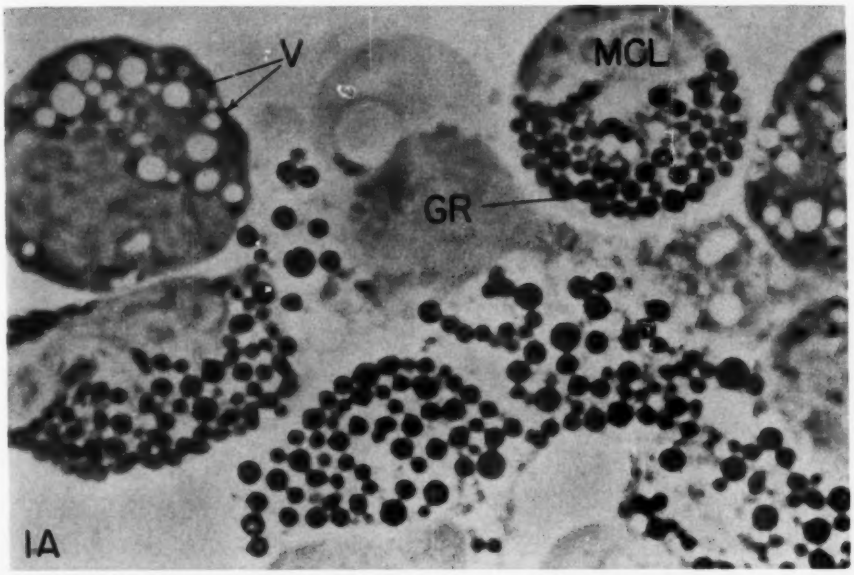
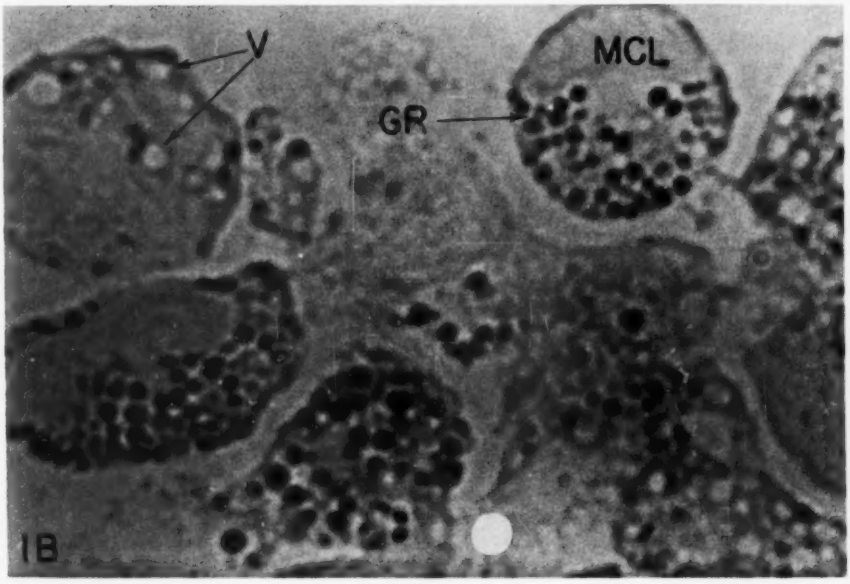
PLATE 177

FIGURE 1A.—Phase-contrast photograph of cells from normal bone marrow with myelocytes (MCL) containing granules (GR). Appearance of vacuoles is indicated at (V). × 3,200

FIGURE 1B.—Same cells showing positive adenosinetriphosphatase reaction of granules (GR) of myelocytes (MCL) after freeze-drying and fixation with formaldehyde. × 3,200



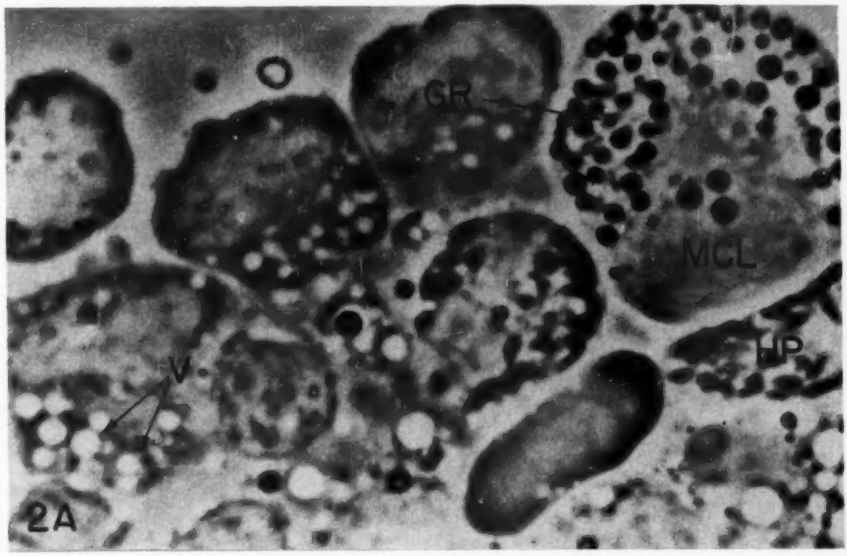


567420—60——23

PLATE 178

FIGURE 2A.—Phase-contrast photograph of cells like those of figure 1A including heterophils (HP). × 3,200

FIGURE 2B.—Same cells after application of freeze-substitution with acetone and staining for adenosinetriphosphatase activity. Reaction of granules was essentially negative except for those in the unidentified cell at *upper left*. × 3,200


GR
MCL
HP
V
2A

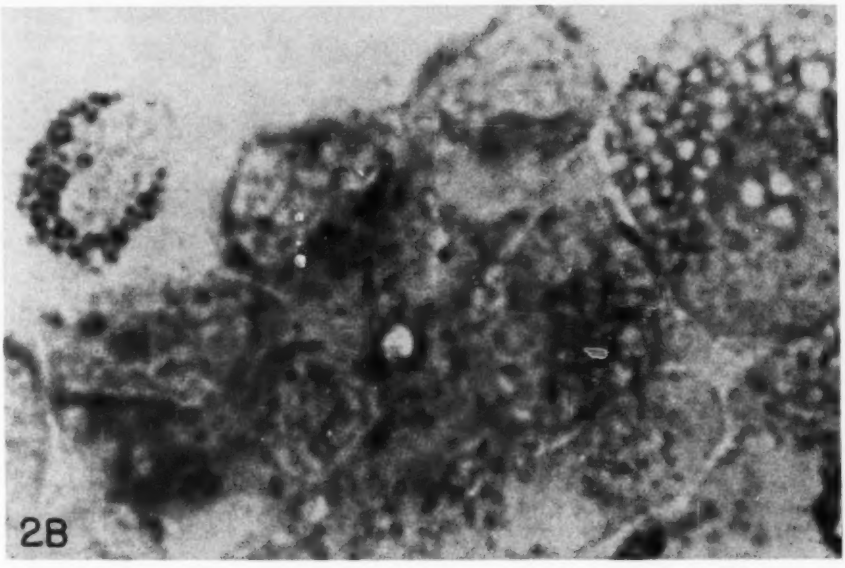

2B

PLATE 179

FIGURE 3A.—Phase-contrast (negative) photograph of virus-associated myeloblasts from tissue culture damaged by pressure as indicated in part by separation of the cytoplasm from the nucleus (R). × 2,800

FIGURE 3B.—Positive adenosinetriphosphatase reaction of the mitochondria (M) of the damaged cells. Fixation as in figure 2B. × 2,800

FIGURE 4A.—Phase-contrast photograph of virus-associated myeloblasts from tissue culture showing viroplasts (VPL), vacuoles (V), and Golgi region (G). × 3,200

FIGURE 4B.—Same cells frozen, dehydrated and stained for adenosinetriphosphatase activity without prior fixation with formaldehyde. Both viroplasts (VPL) and mitrochondria are deeply stained, and a diffuse staining is seen in the Golgi region (G). × 3,200

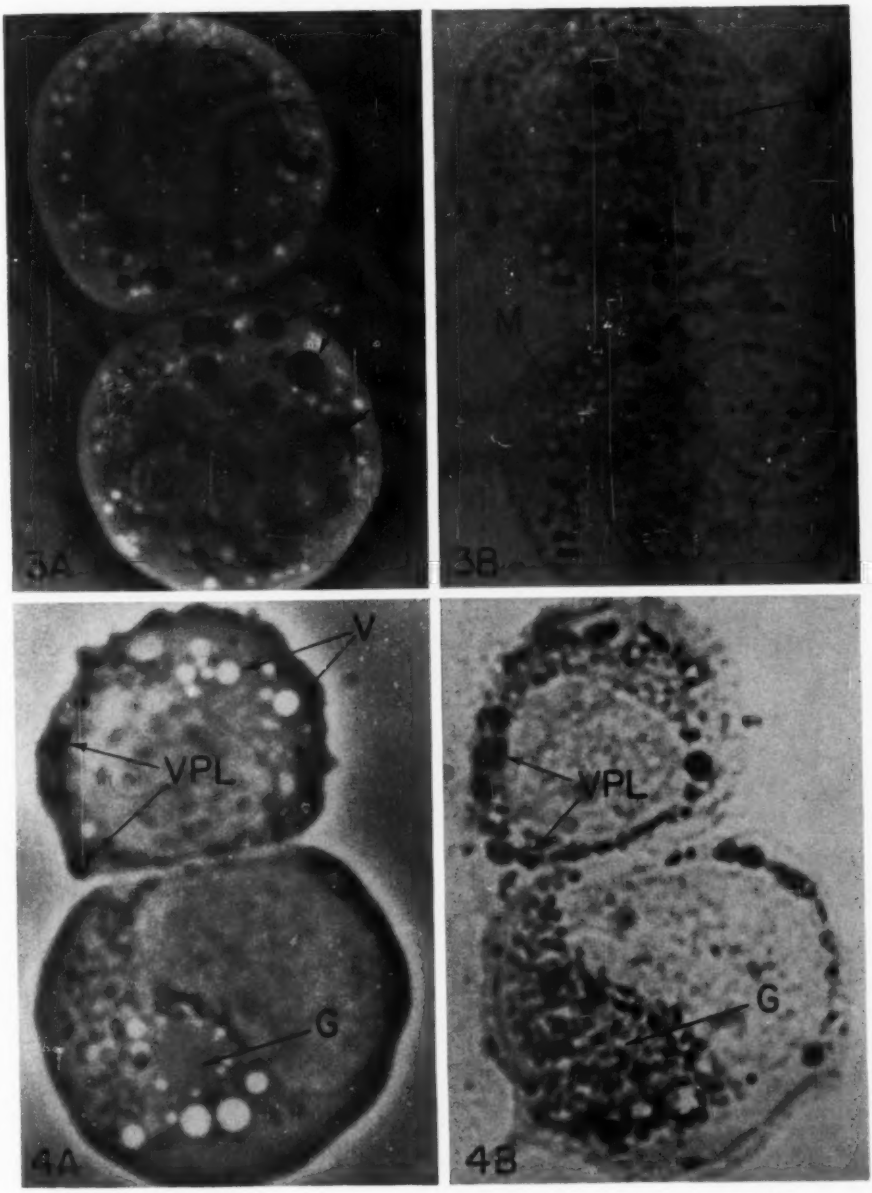

3A
3B
V
VPL
G
4A
VPL
G
4B

PLATE 180

FIGURE 5A.—Phase-contrast photograph of myelocytes (MCL) and heterophils from normal bone marrow. × 2,200

FIGURE 5B.—Positive inosinetriphosphatase reaction of the granules of the same cells after freeze-drying and fixation with formaldehyde. × 2,200

FIGURE 6A.—Phase-contrast photograph of virus-associated myeloblast from tissue culture showing vacuoles (V) and viroplasts (VPL). × 4,000

FIGURE 6B.—Strongly positive staining for inosinetriphosphatase activity in viroplasts. Fixation as in figure 5B. × 4,000

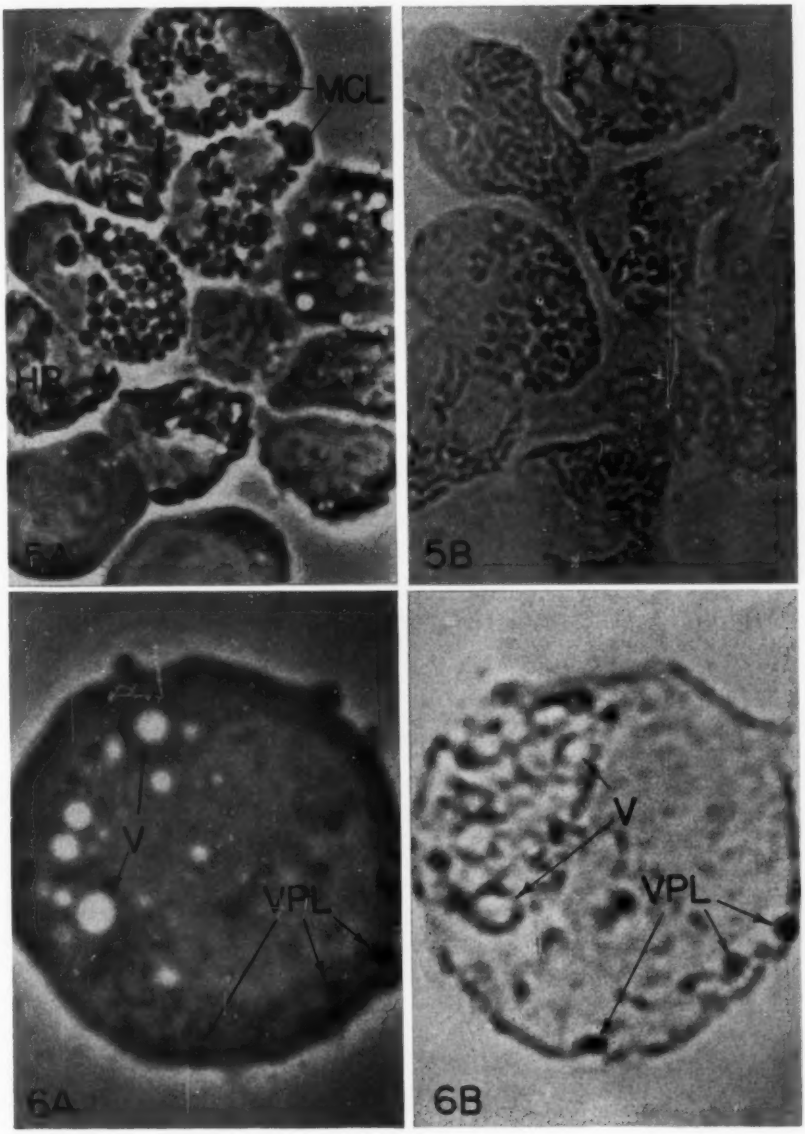

MCL
HR
5A
5B
V
VPL
6A
V
VPL
6B

PLATE 181

FIGURE 7A.—Phase-contrast photograph of cells from normal bone marrow. × 2,700

FIGURE 7B.—Positive adenosinediphosphatase reaction of granules of the same cells. Fixation as in figure 5B. × 2,700

FIGURE 8A.—Phase-contrast photograph of virus-associated myeloblasts from tissue culture showing viroplasts (VPL). × 2,700

FIGURE 8B.—Positive adenosinediphosphatase reaction of viroplasts. Fixation as in figure 5B. × 2,700

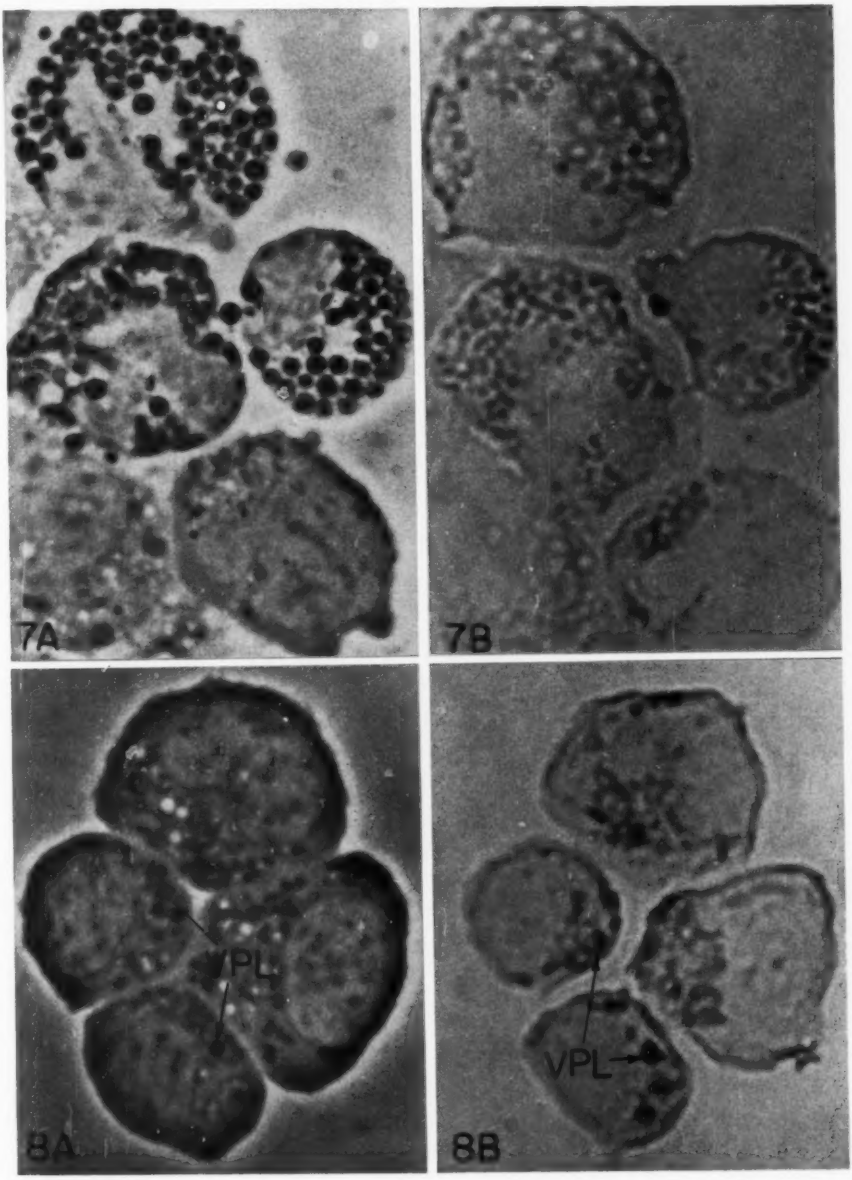

7A
7B
VPL
8A
VPL
8B

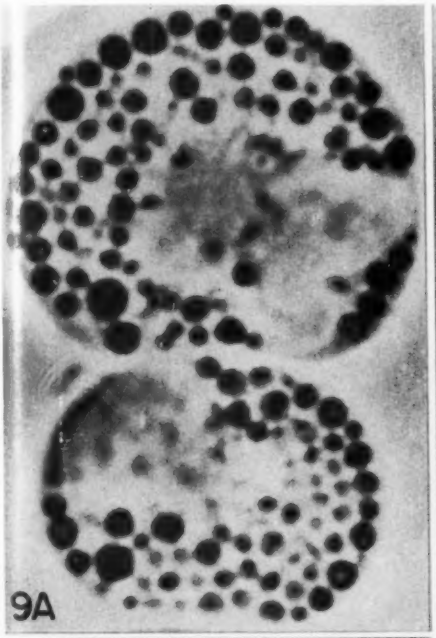
PLATE 182

FIGURE 9A.—Phase-contrast photograph of myelocytes of normal bone marrow. × 3,500

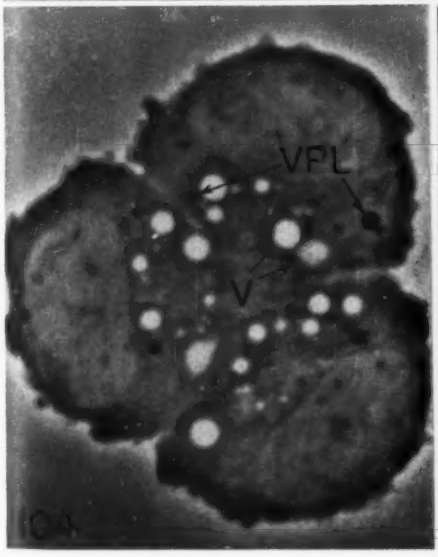
FIGURE 9B.—Complete lack of reaction of granules of the same cells with adenylic acid as the substrate in the Wachstein-Meisel procedure. Fixation as in figure 5B. × 3,500

FIGURE 10A.—Phase-contrast photograph of virus-associated myeloblasts from tissue culture showing viroplasts (VPL). × 2,800

FIGURE 10B.—Same cells treated as those in figure 9B showing no reaction of viroplasts in presence of adenylic acid as substrate. × 2,800


9A

9B


VPL
V
10A

10B

PLATE 183

FIGURE 11A.—Phase-contrast photograph of virus-associated myeloblasts from tissue culture showing viroplasts (VPL). × 2,400

FIGURE 11B.—Negative reaction of viroplasts in alkaline-phosphatase test. × 2,400

FIGURE 12A.—Phase-contrast (negative) photograph of virus-associated myeloblast after 6 days in tissue culture in presence of 5-methyl tryptophan. Picture illustrates great number of viroplasts (VPL) of very large size characteristic of cells under these conditions. × 4,000

FIGURE 12B.—Viroplasts (VPL) of the same cell strongly stained for adenosine-triphosphatase activity. Fixation by freeze-substitution with acetone. × 4,000

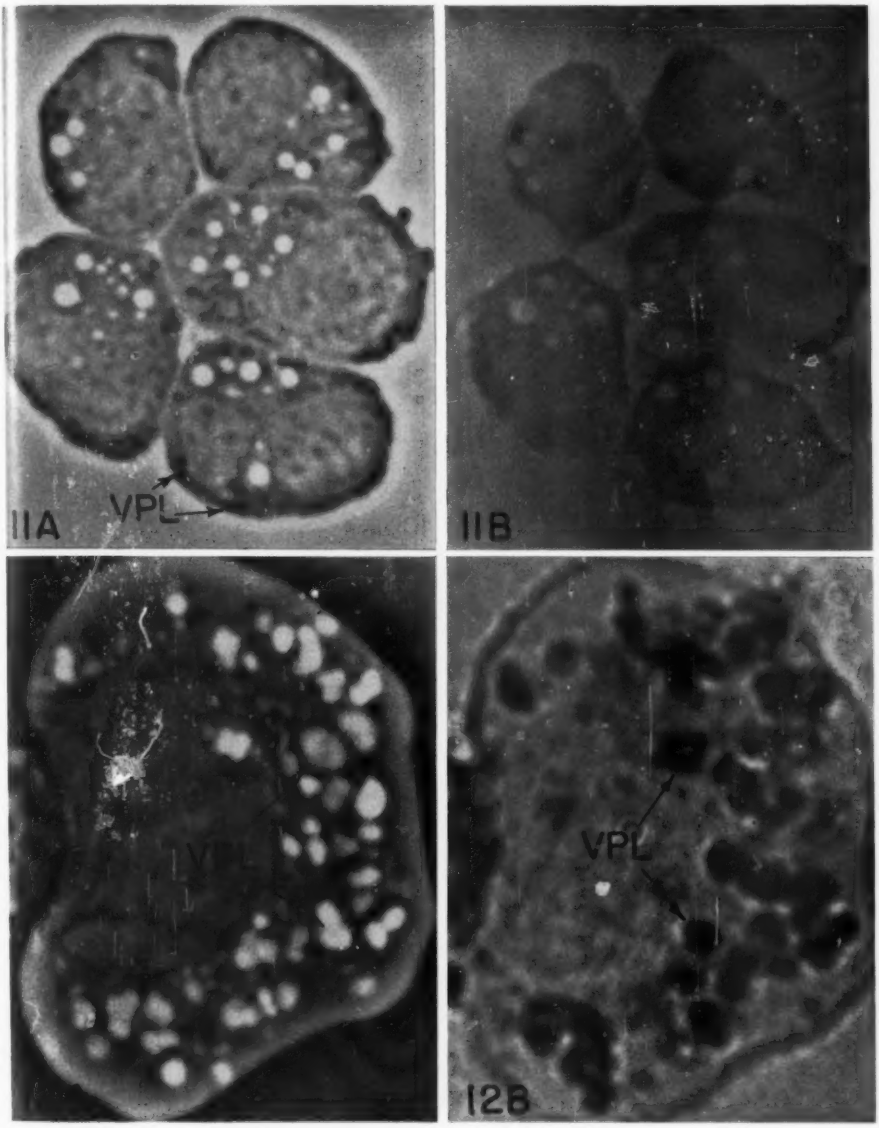

11A
VPL
11B
12B
VPL

PLATE 184

FIGURE 13.—Electron micrograph of a virus-associated myeloblast from tissue culture containing 5-methyl tryptophan (*see* fig. 12). Cell is crowded with viroplasts (VPL) of typical appearance, one containing a virus particle (VP). Others show circular images of structures (S) which may be related to synthesis of the virus. Many mitochondria (M) are of normal appearance. × 40,000

M
V-L
M
3

Reducing Bodies in the Adrenal Medulla Fixed With Zenker-Formol [1]

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SUMMARY

The adrenal medulla fixed in Helly's fluid (Zenker-formol) reveals hematoxyphilic particles. Histochemical studies of these strongly reducing particles indicate they are composed of catechol amine. Spontaneous mouse pheochromocytomas fixed in Helly's fluid show similar reducing bodies conspicuously.—J. Nat. Cancer Inst. 25: 1451–1459, 1960.

IN EXAMINING the adrenal glands of mice and rats fixed in Zenker-formol (Helly's fixative) and stained with hematoxylin and eosin, we regularly observed small, amorphous masses of brownish-blue material in the medulla. At a magnification of 100× these bodies varied from a barely perceptible, shadowy fleck to irregular masses of 10 μ diameter. They showed no constant relationship to any structures within the medulla. These particles were found in the cytoplasm, often at cell borders, and sometimes they appeared in intercellular spaces or within vascular lumens (fig. 1). The properties and prevalence of these medullary bodies are the subject of the present investigation. The flecks or small, round bodies somewhat similarly stained by hematoxylin and eosin, which have been observed within the cytoplasm of adrenocortical cells in the zona fasciculata, will be considered separately (*1*).

The only description we have found that seems to relate to these bodies appeared in a monograph by Selye and Stone on the experimental morphology of the adrenal cortex (*2*). In the adrenocortical cells of the rat, they noted granules having the staining properties of nuclear chromatin, and "for lack of a better term," they referred to these granules as "chromidia." The authors stated that the development of these granules was so irregular that they might even be postmortem artifacts, yet "even in this event, they would be of interest in connection with the morphology of the adrenals, since they rarely occur in other organs similarly prepared for histologic study." The granules occurred "not

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only in cortical, but also in adrenal medullary cells," though, generally, the basophilic granules in the cytoplasm of cortical cells were "fine and dust-like," while those of the medulla were "coarse and granular."

The authors further discussed the possible derivation of these granules from the nucleus, but stated later that their observations would be equally compatible with the assumption that the material was produced in the cytoplasm itself, or invaded the cytoplasm from the blood stream. Because of the importance of chromatin-like materials in the function of the cell, they felt that these structures should be carefully examined since "they do not appear to have received attention up to the present time." The photographs illustrating these "chromidia" appear to us to be similar to the "bodies" we have observed. We could find no statement regarding the fixative used by Selye and Stone.

We have been unable to find any publication in which a more precise description of similar bodies has been given or in which an investigation of the occurrence and histochemistry was undertaken. We are, therefore, reporting the results of a histochemical study and a survey of a number of rodents, under normal and pathological conditions, during which observations were made on the bodies in the adrenal glands.

METHODS

Tissues from rodents obtained immediately post mortem and human surgical specimens were fixed as specified prior to routine paraffin embedding and sectioning at 5 μ. Fixative solutions were prepared and histochemical procedures were carried out as outlined in detail by Lillie (*3*). The ferric chloride-ferricyanide procedure of Golodetz and Unna (*4*), as modified by Lillie, involved a 10-minute exposure of rehydrated sections in an aqueous solution containing 0.1 percent $K_3Fe(CN)_6$ and 0.75 percent $FeCl_3$. For morphologic study, sections were stained with Mayer's hematoxylin followed by eosin (*3*).

RESULTS

Histochemical tests.—The medullary hematoxyphilic bodies in Helly-fixed adrenals colored dark blue-green with the ferric chloride-ferricyanide test for reducing groups [(*4*), fig. 2]. Chromaffin in the dichromate-fixed adrenal stained similarly [(*5*), fig. 3]. Dark-green particles were frequently seen immediately outside the medulla at the corticomedullary border.

The medullary bodies appeared to contain little or no polysaccharide, nucleic acid, lipide, protein, or ferric iron, since they failed to color with the periodic acid-Schiff method, alcian blue, azure A, the Feulgen procedure, Sudan black B, the Smith-Dietrich method, eosin B, procedures

for protein-bound tyrosine and tryptophane, and the ferrocyanide-Prussian blue method, respectively. They failed to azocouple with stable (Garnet GBC) or freshly prepared (diazo safranin) diazotates in the *p*H range 5.5 to 8.5. They also failed to react with ferric chloride in the Vulpian reaction and lacked argentaffin reactivity with methenamine or diammine silver.

Fixation

The influence of fixative solution on preservation of both the hematoxyphilic-reducing particles and the chromaffin substance has been investigated. Twenty-four hour fixation in solutions containing 7 percent $HgCl_2$ and 2.5 percent $K_2Cr_2O_7$, with or without included 10 percent formalin, preserved the particles optimally and the chromaffin substance partially. Retention of the bodies required inclusion of both the mercuric salt and dichromate in the fixative. Forty-eight hour fixation in Kose's fluid containing 2.7 percent dichromate and 10 percent aqueous formalin preserved chromaffin optimally (fig. 3). Bouin's fluid (fig. 4), neutral formalin, and alcoholic fixatives preserved neither entity. Nor were ferric-ferricyanide reactive chromaffin substance or medullary bodies demonstrable after a 40-hour fixation with a sodium acetate buffered 7 percent $HgCl_2$ solution in 10 percent formalin, or a solution of 10 percent formalin containing 7 percent $HgCl_2$ + 1 percent HIO_4, or $KMnO_4$, or 0.5 percent OsO_4, or 2 percent $NaIO_3$.

For the clearest demonstration of the reducing particles, mercurial precipitates were removed from the tissues before staining. This was done by a 5-minute exposure to a solution containing 0.2 percent I_2 and 0.4 percent KI in water, followed by 2 minutes in aqueous 5 percent $Na_2S_2O_3$ (*4*). In sections from which mercury was not removed the precipitates occupied the same sites as the reducing bodies. The random mercurial precipitates commonly seen throughout all Helly-fixed tissues showed no coloration with the reducing test.

Morphology

The ferric chloride-ferricyanide stained bodies were light or dark blue-green. The size varied from 2 to 10 μ, and they were globular, irregular, or occasionally stellate in shape. They often assumed a semilunar configuration around the nucleus. Frequently globules lay in the vascular lumens (fig. 5), and those in the adjacent cortex appeared in some instances to be located between cells (fig. 6). With Helly's fixation the cytoplasm of some chromaffin cells stained relatively weakly in the ferric-ferricyanide method, but these, as well as darker stained cells, contained reducing bodies.

Model Test

An *in vitro* model was used in an effort to identify the reactive material in the reducing medullary bodies through comparison with authentic

standards. Test materials were injected post mortem into the liver, kidney, intestine, and skeletal muscle of the mouse, and blocks of tissue containing the injected area were fixed in Helly's fluid before routine embedding and staining by the ferric chloride-ferricyanide method. When aqueous suspensions of epinephrine or norepinephrine were injected in this way, a diffuse, dark blue-green coloration, most intense toward the center, was observed in the injected area of each organ. In addition, as illustrated for skeletal muscle in figure 7, there were numerous blue-green bodies in each injected area similar to those occurring naturally in the adrenal medulla. The bodies, after injection, were found in the stromal septa, intercellular spaces, and vascular lumens over an extensive area surrounding the site of injection. They appeared brown in hematoxylin- and eosin-stained sections.

Physiological Occurrence

Morphologically similar bodies were visualized in the adrenal medulla of all species examined, including the mouse, rat, guinea pig, rabbit, and man. In rodents no variation in the number of forms of the particles was detected in correlation with the age or sex of the animal. A survey of numerous organs and tissues of the mouse, with the use of hematoxylin and eosin as well as the ferric chloride-ferricyanide stain on Helly-fixed tissue, revealed no other particulates similar to those observed in the adrenals. The organs and tissues examined were brain, medulla, spinal cord, nerve ganglia and fibers, the gastrointestinal tract, the reticuloendothelial system, the heart, lungs, kidney, skeletal muscle, eye, the reproductive system, and other endocrine organs, including thyroid, parathyroid, pituitary, and pineal glands.

The influence of reserpine on the chromaffin substance in the dichromate-formalin-fixed adrenal was compared with its effect on the reducing bodies in the contralateral, Helly-fixed organ. A subcutaneous dose of 1 mg of reserpine in adult male rats depleted the chromaffin substance in 24 hours, as expected (*6*), and also affected the disappearance of the reducing bodies. Two days after injection, both the chromaffin substance and the reducing bodies were found in about the same quantity as in untreated rats.

Medullary Bodies in Pathological Conditions

Helly-fixed tissue blocks from both of two spontaneous mouse adrenal pheochromocytomas, kindly provided by Dr. Elizabeth Jones (*7*), revealed numerous reducing bodies morphologically like those in the normal medulla (figs. 8 and 9). These varied in different areas of the tumors from small globules to larger, irregular bodies. Dr. Clyde Dawe (*8*) provided us with Helly-fixed specimens of adrenal tumors resulting from treatment of the newborn mouse with the parotid-tumor agent. These failed to show reducing particles. We also searched for the bodies in spontaneous adrenocortical tumors in the mouse and in one from the rat (*9*) and failed to find them.

Adrenal glands were examined from various groups of mice. The ferric chloride-ferricyanide procedure was used on material previously fixed in Helly's fluid. In old BALB/c mice showing extreme "brown degeneration," no reducing bodies were found in the characteristic cells. They were abundant, however, in the adrenal medulla of mice bearing an adrenocortical tumor that produced extreme atrophy of the adrenal cortex of the host mouse. Small particles were sometimes seen in the X zone that appears at the corticomedullary junction in young mice. It is probable that these were derived from the nearby medulla and were not developed by X-zone cells.

DISCUSSION

Although conspicuously stained by the ferric-ferricyanide method, the medullary bodies are less prominent in hematoxylin-stained sections. This, plus the relatively restricted use of Helly's fixation, may account for the lack of previous studies on the medullary bodies.

The available evidence indicates that the material demonstrated is chemically related to or identical with the catechol amines. Thus its strong reducing property in common with adrenal chromaffin and its exclusive occurrence in the adrenal medulla point to this conclusion, as does observation of similar morphologic aggregates in the area of injected epinephrine and norepinephrine. The evident conclusion is that chromaffin material, present as a diffuse precipitate in dichromate-formalin-fixed tissue, aggregates in discrete particles when mercury salts are included.

The mechanism of reduction of iron salts by the adrenal bodies remains unclear. The requirement for both the mercurial and dichromate in the fixative solution complicates the interpretation. On the assumption that catechol amine is visualized, possibly adrenochrome or some other early oxidized form of the amine precipitates with the mercuric salt. The reducing bodies obviously coincide with microscopically visible mercurial precipitate when viewed in nondezenkerized sections. *In vitro* tests show that adrenaline in solution does not precipitate with $HgCl_2$ alone as it does with $K_2Cr_2O_7$ or $K_2Cr_2O_7$ plus $HgCl_2$. Apparently the morphology of the precipitate of catechol amine with dichromate in tissues differs when the mercury salt is included.

Either the insolubilized, probably polymerized, and perhaps incompletely oxidized form of the catechol amine itself reduces the iron mixture, or a reduction product of the dichromate precipitated at the side of oxidation of the catechol amine reacts to reduce ferric iron. That the reduction may be mediated by the reduced product from the dichromate is indicated by the observation that other oxidants, such as permanganate, osmic acid, iodate and periodic acid, fail to substitute for the dichromate. However, periodate and permanganate probably oxidize adrenaline more completely than does dichromate. Slight evidence for the presence of

chromium salt in the medullary reducing particles is their hematoxyphilia, which could be explained on the basis of chelation of the dye with the metal.

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PLATE 185

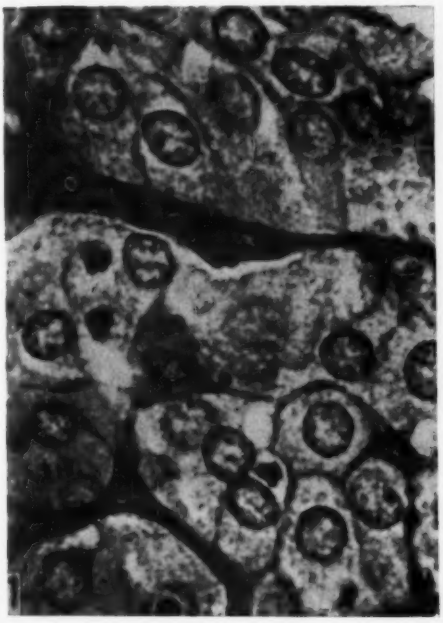
FIGURE 1.—Hematoxyphilic bodies in adrenal medulla of the mouse. Bodies of irregular size and shape in no particular relationship to any cell element. One body lies in the center of a nucleus, others are scattered in the cytoplasm, or at cell borders. Hematoxylin and eosin, after Helly's (Zenker-formol) fixation. × 950

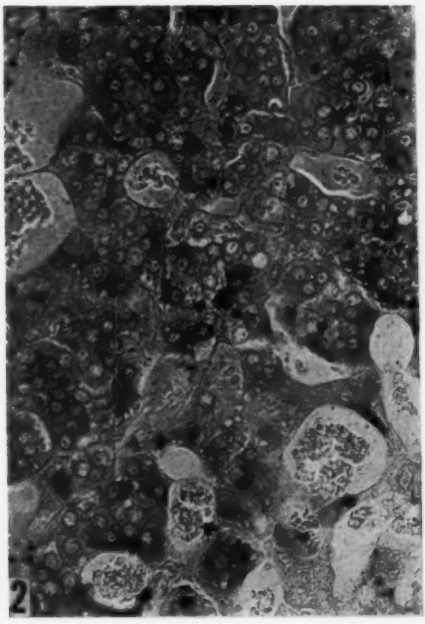
FIGURE 2.—Reducing bodies morphologically similar to the hematoxyphilic bodies plus chromaffin substance in mouse adrenal medulla, contrasting with unstained cortex at *lower right*. Ferric-ferricyanide stain after Helly's fixation. × 310

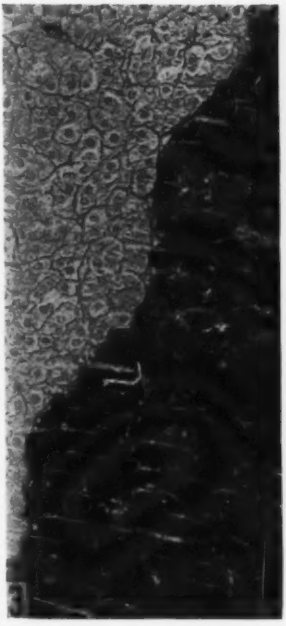
FIGURE 3.—Diffuse chromaffin substance in mouse adrenal medulla on *right*. Ferric-ferricyanide after Kose's (dichromate-formol) fixation. × 205

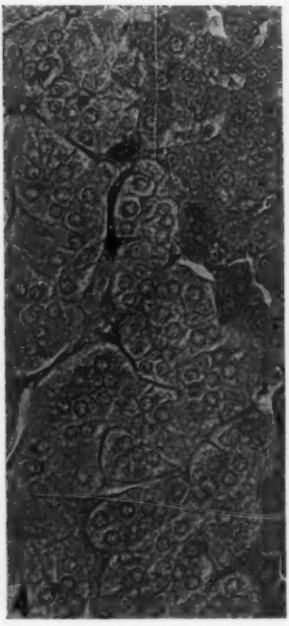
FIGURE 4.—Mouse adrenal medulla, on *left*, lacking reducing bodies or chromaffin substance. Ferric-ferricyanide after Bouin's fixation. × 310

FIGURE 5.—Reducing bodies in cells and vascular lumen, and diffuse chromaffin substance in cells of mouse adrenal medulla. Cortex in *right upper corner* is unstained. Ferric-ferricyanide after Helly's fixation. × 205









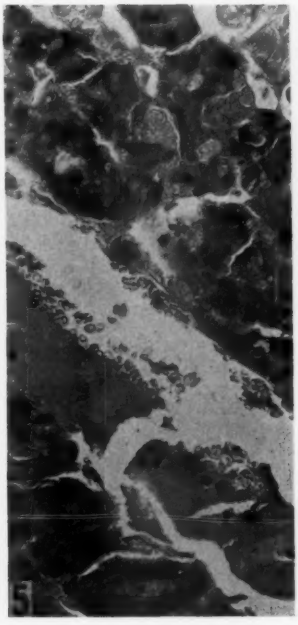


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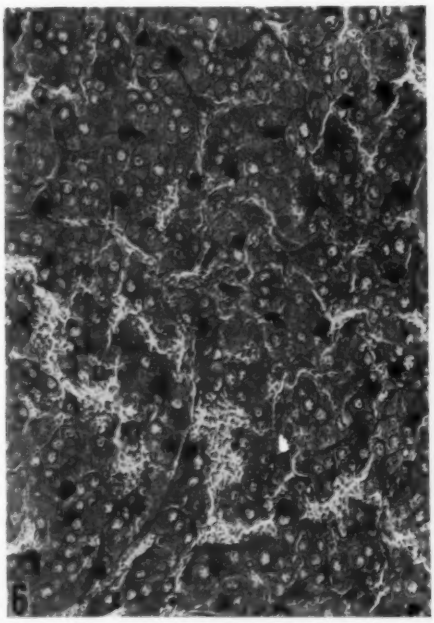
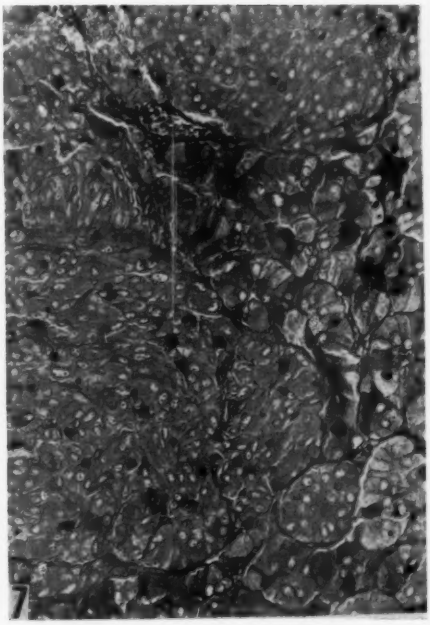
FIGURE 6.—Reducing particles in zona reticularis and medulla of mouse adrenal and diffuse chromaffin substance. Ferric-ferricyanide after Helly's fixation. × 205

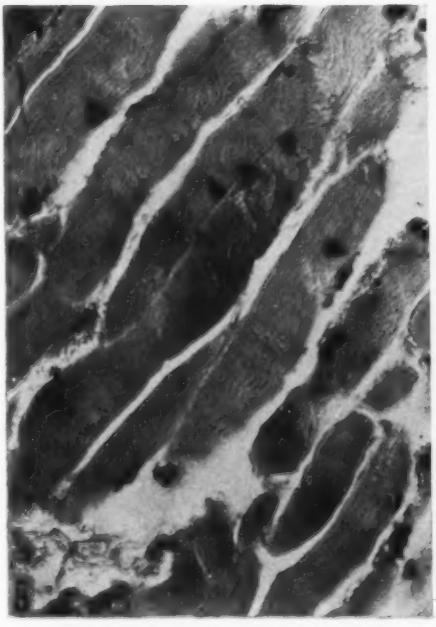
FIGURE 7.—Reducing particles near site of postmortem injection of adrenalin in mouse skeletal muscle. Ferric-ferricyanide after Helly's fixation. × 310

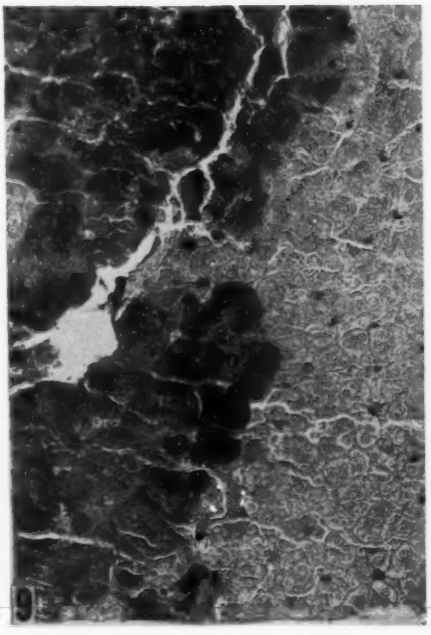
FIGURE 8.—Reducing bodies in cells of mouse pheochromocytoma. Ferric-ferricyanide after Helly's fixation. × 200

FIGURE 9.—Reducing bodies in a second mouse pheochromocytoma. Ferric-ferricyanide after Helly's fixation. × 235









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"Proliferation of mammalian cells in ion-controlled environments," by John L. Worden.

"The antigens of human leukocytes. I. Introduction," by Leonhard Korngold, Gerda van Leeuwen, and Daniel G. Miller.

"The antigens of human leukocytes. II. The specificity of leukocyte antigens," by Leonhard Korngold, Gerda van Leeuwen, and Daniel G. Miller.

"Regression of grafted bone marrow in homologous irradiated mouse chimeras," by Raymond A. Popp.

"Cytotoxic action *in vitro* of human serum on the metabolism of mouse ascites cells, and counteraction by endotoxic and other polysaccharides," by Berigoj Stambuk and Dean Burk.

"Effects of feeding thiouracil on the thyroid glands of rats," by Seymour H. Wollman.

"Chinese hamster cell strains *in vitro:* spontaneous chromosome changes and latent polyoma-virus infection," by Denys K. Ford, Christina Boguszewski, and Nelly Auersperg.

"Virus of avian erythroblastosis. VII. Ultrastructure of erythroblasts from the chicken and from tissue culture," by Ursula Heine, G. S. Beaudreau, Caroline Becker, Dorothy Beard, and J. W. Beard.

"The rabbit heart cell culture (strain RHF–1). I. Growth, cytology, histology, and susceptibility to some inclusion inducing viruses," by Newton Khoobyarian and Catherine Gardella Palmer.

"Modification of characters of an *in vitro* cell line after an increase of its tumor-producing capacity," by Georges Barski, June L. Biedler, and Fr. Cornefert.

"A simple method for the preparation of potent chicken anti-Rous sarcoma virus serum," by Mary Alexander Fink and Frank J. Rauscher.

"The effect of phenazine di-*N*-oxide and phenazine on the total cellular dry mass of mouse Ehrlich ascites cells by interference microscopy," by Hun Lee, Victor Richards, and Arthur Furst.

"Dissemination of Rous sarcoma virus as a cause of 'metastases,'" by J. Spencer Munroe and Chester M. Southam.

"Enzymatic deamination of deoxyadenylic and adenylic acids by normal and cancerous rat liver tissues," by Silvio Fiala and Harold E. Kasinsky.

"Antigenicity of pooled human malignant and normal tissues by cytoimmunological technique: III. Distribution of tumor antigen," by Bertil Björklund, Viveka Björklund, and Ingegerd Hedlöf.

"Cytotoxicity of heterologous immune chicken serum on normal and neoplastic mouse tissues in culture," by Peter Abramoff, John W. Saunders, Jr., and Mary T. Casseling.

"Induction of tumors in newborn mice with dibenz[*a*,*h*]anthracene and 3-methylcholanthrene," by Margaret G. Kelly and Roger W. O'Gara.

"On pathogenicity of the Rous sarcoma virus for rats and rabbits," by L. A. Zilber.

"On the interaction between tumor viruses and cells (a viro-genetic concept of tumor genesis)," by L. A. Zilber.

"Studies of the properties of the liver mitotic stimulant (LMS) in mouse tumor tissue," by Richard A. Malmgren and Willie Mills.

"Tumorigenesis in transplanted irradiated and nonirradiated ovaries," by W. U. Gardner.

"Tumors of the central nervous system of rats: With report of two tumors of the spinal cord and comments on 'posterior paralysis,' " by J. R. M. Innes and G. Borner.

Symposium on Fundamental Cancer Research

"The Molecular Basis of Neoplasia" is the theme of the fifteenth annual symposium on fundamental cancer research, sponsored by The University of Texas M. D. Anderson Hospital and Tumor Institute, February 23, 24, and 25, 1961.

Sessions of the meeting will be devoted to nucleic acids, nucleic acids and proteins, mutation and protein structure, ribosomes and protein synthesis, controlling mechanisms and enzyme synthesis, and biochemical alterations induced by viral nucleic acids.

Information on the symposium may be obtained by writing the Publications Department, The University of Texas M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston 25, Texas.

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